STUDIES ON THE GENETIC REGULATION OF MYCOPARASITISM IN *TRICHODERMA HAMATUM*

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by Johanna Steyaert

The ability of *Trichoderma* species to mycoparasitise other fungi as a source of carbon has led to their exploitation as biocontrol agents of soilborne fungal diseases, however widespread implementation has been hampered by inconsistent field performance. Usage recommendations based on field studies have provided a more consistent product, by optimising conditions for biocontrol. Genetic studies on genes implicated in mycoparasitism have revealed molecular targets for optimising biocontrol response, however much is unknown about the genetic regulation of mycoparasitism. The majority of reported *Trichoderma* biocontrol agents are *T. harzianum* or *T. atroviride*, however a *T. hamatum* isolate (6Sr4) with significant activity against *Sclerotinia* diseases of vegetable crops, has recently been developed for use as a commercial biocontrol agent of soilborne fungal diseases. In this study, three genes previously implicated in the mycoparasitic ability of *T. atroviride* were characterised from 6Sr4, and from two other *T. hamatum* isolates of variable biocontrol potential to further current knowledge on the regulation of mycoparasitism, and identify molecular targets for research on optimisation of *Trichoderma* biocontrol activity in the field.

Using a combination of targeted, degenerate and inverse PCR, gene orthologues of *chit42*, *prb1* and *xbg1.3-110* were isolated, cloned and sequenced from all three *T. hamatum* isolates and sequence analysis performed on the regulatory regions. Comparison between the different genes revealed regions of identity between *chit42* and *prb1*, which supported the existence of a global inducer of mycoparasitism, suggested for *T. atroviride*. Identification of putative regulatory motifs in *prb1* supported mutational work in *T. atroviride prb1*, which suggested an actively bound GATA site involved in nitrogen repression.

Northern blotting was performed to examine gene expression under alternate carbon sources and during mycoparasitism. In 6Sr4, *chit42* and *prb1* were strongly induced under high glycerol, whereas no induction was detected from *xbg1.3-110*. Conversely, no *chit42* or *prb1* induction was detected from a *T. harzianum* isolate (JD2) grown in the same study

and only low induction of *prb1* from *T. atroviride* has been reported. This suggested *T. hamatum chit42* and *prb1* to be under control of an additional pathway not active in *T. harzianum* and *T. atroviride.*

Both *chit42* and *prb1* were moderately induced during confrontation against *Sclerotinia sclerotiorum*, which supported a role in mycoparasitism. *Xbg1.3-110* had been previously implicated in mycoparasitism by *T. harzianum*, however no northern analysis during mycoparasitism has not been reported. This study detected no *xbg1.3-110* expression during confrontation and therefore questions its importance in mycoparasitism.

Genetic variation between the three isolates was assessed using UP-PCR, sequence alignments of *chit42*, *prb1* and *xbg1.3-110*, and comparison of *chit42* expression in response to elevated glycerol and during confrontation with *S. sclerotiorum*. 6Sr4 and 3Sr4-2 were more similar than either isolate with S1BYG, which did not correlate with observed biocontrol potential or colony morphology. Sequence analysis revealed multiple single nucleotide polymorphisms (SNPs) within the regulatory regions of *chit42*, *prb1* and *xbg1.3-110*, some associated with putative regulatory sites. In S1BYG, *chit42* expression was delayed relative to 6Sr4 and 3Sr4-2.

These studies identified multiple targets for future research. Most notable is the potential to incorporate glycerol into inoculant amendments to optimise *chit42* and *prb1* expression. It is likely field studies will be conducted to determine amendment effects on biocontrol activity.

Keywords: *Trichoderma hamatum*, mycoparasitism, gene regulation

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Abbreviations

A	adenine
ascn#	accession number
bp	base pairs
C	cytosine
cm	centimetres
d	days
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
dGTP	deoxyguanosine triphosphate
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetra-acetate
ETS	external transcribed spacer
G	guanine
g	gram
gDNA	genomic deoxyribonucleic acid
h	hours
ITS	internal transcribed spacer
kb	kilobase
kDa	kilodalton
L	litre
M	molar
min	minutes
mg	milligram
mol	mole
mL	millilitre
mm	millimetre
mM	millimolar
MM	minimal media
mRNA	messenger ribonucleic acid
N	any nucleotide (A, C, G or T)

ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pers. comm.	personal communication
pg	picogram
pmol	picomole
psi	pounds per square inch
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
s	seconds
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate
T	thymine
Tris	Tris(hydroxymethyl) aminomethane
TSP	transcription start point
U	units
UP-PCR	universally primed polymerase chain reaction
UV	ultraviolet
V	volts
xg	relative centrifugal force
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactosidase
°C	degrees celsius
α	alpha
β	beta
μg	microgram
μL	microlitre
μM	micromolar
μmol	micromoles

Chapter One
Introduction

1.1 Biological Control of Plant Disease

1.1.1 A Definition of Biological Control

The standard definition of biological control provided by Cook and Baker (1983) states, "*Biological control is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man.*" Baker (1987) further extended this definition stating "*... one or more organisms including the host plant...*". In this definition, biological control is represented as a phenomenon rather than the combined activities of various pathologists and biocontrol specialists. This representation is important, as biological control is not merely a product of modern agricultural technology but rather a natural phenomenon important in the biological balance of ecosystems.

A complex series of interactions determine the presence of an organism in a given place and time which, when in balance, determine success for the individual and/or ecosystem (Figure 1.1). A degree of biological control occurs naturally in interactions between a species and antagonists, pathogens and predators in a given environment.

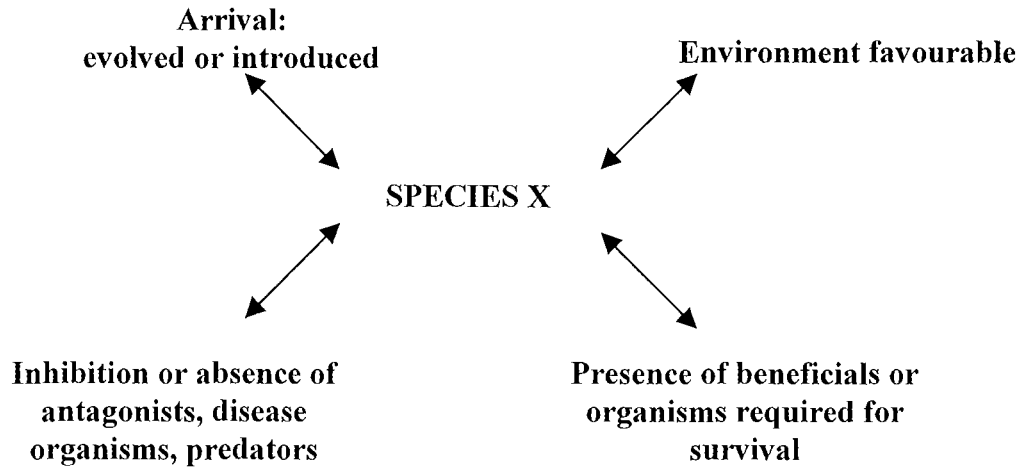


Figure 1.1. Factors determining the presence of a given organism, adapted from Cook and Baker (1983).

1.1.2 Observations in Nature: Suppressive Soils

Observations in nature have lain the foundation of knowledge in biological control. The classic observation of biological control in the environment is the phenomenon of suppressive soils. A soil is considered suppressive when a resident pathogen fails to develop and cause disease. The causes are abiotic and/or biotic in nature, more often involving one or more microorganisms antagonistic to the pathogen (Agrios, 1988).

Suppressiveness has been defined as general or specific. General suppression stems from the combined microbial activity and total soil biomass at a time critical to the pathogen, and is not usually transferable (Baker, 1987). Specific suppression stems from an individual or group of antagonists. Specific suppression has been transferred to a conducive soil, indicating it is of biological origin.

Investigations into the mechanisms operating in suppressive soils have led to a greater understanding of biological control. A combination of competition and antagonism are the underlying mechanisms of specific suppression (Deacon and Berry, 1993). Competition is always present even if it is not the major factor, for example, faster utilisation of the carbon source, or sequestering of nutrients, preventing utilisation by the pathogen. Antagonism occurs through the production of antibiotics and/or parasitism of the pathogen.

Numerous microorganisms have been isolated from suppressive soils and identified as the causal agent in soil suppressiveness to a given pathogen. Of these, many have biological control activity, and some have been commercialised into products (Whipps, 1997). The relative success in isolating antagonists with superior biocontrol activity highlights the value of enhancing and manipulating naturally occurring organisms.

1.1.3 Why Biological Control?

The impetus for developing biological control agents (BCAs) has been the public perception of pesticide toxicity in the environment (Cook and Baker, 1983; Becker and Schwinn, 1993; Deacon and Berry, 1993; Whipps, 1997). Organic (residue free) produce has become a valuable commodity, reflecting consumer fear of the toxic effects of pesticide residues.

Environmental effects have been well documented. Water pollution by pesticides has been recognised as a problem in the United Kingdom since the 1970's. The main focus has been on

the amount of pesticides entering waterways through leaching and run-off (Skinner *et al.*, 1997). The E.C.[1] has stipulated maximum allowable limits of a given pesticide at $0.1\mu g.l^{-1}$. Concentrations ranging from $0.5\ \mu g.l^{-1}$ to $11.5\ \mu g.l^{-1}$ have been reported throughout the U.K. There is a lack of epidemiological data on humans, however pesticide spray drift causes measurable short-term impacts to wildlife including mammals.

The harmful effects of some individual pesticides, such as methyl bromide, have been documented. Methyl bromide is used as a fumigant to eliminate soil pests and pathogens. It is one of the five most used pesticides in the U.S.A. (Ristaino and Thomas, 1997). Annual application ranges between 25,000 and 27,000 tonnes, the majority of which is released to the atmosphere. In the atmosphere it breaks down ozone in a similar manner to chlorine, however bromine is 50X more reactive as it reacts with reservoir chlorine species freeing them up to react with ozone. Production of methyl bromide will be phased out under international agreements by 2010.

Chemical control of soil-borne diseases has never achieved the same level as that of foliar diseases. Most modern fungicides have been selected for activity on foliar pathogens and although subsequent screening may have revealed potential as a soil fungicide, few were developed solely for this purpose (Becker and Schwinn, 1993). Chemical control is inadequate for many soil-borne pathogens, including *Gaeumannomyces graminis* f.sp. *tritici* (Take-all of wheat) and *Plasmodiophora brassicae* (Clubroot of crucifers). The focus of biological control research has been on soil-borne pathogens, which is not surprising given the urgent need for control regimes.

Pesticide resistance has already evolved within plant pathogen populations. The specific nature of synthetic fungicides has promoted the development of resistance, as the more specific the effect on a microorganism, the greater chance of resistance emerging through genetic shifts in the population (Cook and Baker, 1983). Biological control is usually achieved through a combination of factors reducing the likelihood of a pathogen developing resistance.

1.1.4 The Commercial Reality of Biological Control

A major criticism of biological control is the lack of reproducibility in results. Whipps (1997)

[1] 1980. Official Journal, EEC 80/778, EECL.229. Council Directive on the quality of water for human consumption

cited a failure to consider and study the BCA in an ecological context as the cause. Rather than focusing on one interaction with the pathogen, all interactions need to be included in assays, for example strong *in vitro* antibiosis does not necessarily correlate to high disease protection when antagonist, pathogen and host are present in the same assay.

Despite the criticisms, numerous bacteria and fungi have potential biological control activity against soil-borne pathogens. Reports of successful use of fungal antagonists are dominated by *Trichoderma* spp. and *Gliocladium virens*. These organisms represent one third of all microbial species used in biological control of plant diseases (Chernin & Chet, 2002). Commercial products included GlioGard, BINAB-T, ANTI-FUNGUS, Trichodermin (www.usda.gov), and the New Zealand products Trichodowels, Trichoject, Trichoseal, Trichodry and Trichopel (Alison Stewart, pers. comm.).

Biological control is often recommended as part of an integrated pest management (IPM) programme, where BCAs, pesticides and cultural practices are combined to achieve crop protection.

1.2 The Genus *Trichoderma*

1.2.1 Taxonomy

Trichoderma spp. are anamorphs of the ascomycete order Hypocreales. The majority of the few proven teleomorphs are of the genus *Hypocrea*, however two other Hypocreales genera (*Podostroma* and *Sarawakus*) have *Trichoderma* anamorphs (Samuels, 1996).

The taxonomy of *Trichoderma* is confusing, complex and incomplete (Samuels, 1996; Gams and Bissett, 1998; Kindermann *et al.*, 1998). Species identification has traditionally been based on morphological and cultural characteristics, and while these are still the primary means of identification, many considered them inadequate for correct identification (Samuels, 1996; Kindermann *et al.*, 1998). The literature contains multiple examples of misidentification, including some of the most well characterised isolates (Kullnig *et* al, 2001). The first attempt at a morphological taxonomy was by Rifai (1969), who described *Trichoderma* spp. in terms of nine aggregate species. A more detailed study by Bissett (1991) identified species in terms of five sections, and also included some species originally considered *Gliocladium*. With the advent of molecular taxonomic techniques, the genus is has again been redefined, some morphological sections merged, and even suggestion of replacing morphological sections with

phylogenetic groupings (Kindermann *et al.*, 1998).

For the sake of simplicity, one taxonomy has been presented here. It is based on Bissett (1991), but differs by the mergence of sections *Longibrachiatum* and *Saturnisporum* (Table 1.1) (Gams and Bissett, 1998). Various morphological characters are used to differentiate *Trichoderma* spp. into sections and species, some of which are included in Table 1.1, however it is important to note these descriptors are not absolute, and correct identification should be by a combination of morphological and genetic characters. In general, *Trichoderma* spp. in culture exhibit rapid growth and produce abundant powdery, green conidia formed from phialides (Figure 1.2) (Samuels, 1996).

1.2.2 Ecology and Physiology

Trichoderma spp. are ubiquitous soil dwellers found in all climatic zones. They have been found in agricultural, grassland, forest, saline and desert soils, and are prevalent in the humic layer of hardwood forests where they represented up to 3% of all fungal propagules (Papavizas, 1985; Klein and Eveleigh, 1998). Some species distribution occurs according to environmental parameters, for example *T. viride* is isolated mainly from cool regions whereas *T. harzianum* is characteristic of warm climates. Insensitivity to high pH and low soil moisture content has also been observed.

Trichoderma spp. are fast growing saprophytes able to utilise a wide variety of substrates for growth (Klein and Eveleigh, 1998). Nitrogen sources include ammonium compounds, amino acids and proteins. Carbon sources include general sugars, and fungal polysaccharides which they attack enzymatically. Several strains degrade hydrocarbons and various xenobiotic pesticides including DDT, aldrin and dieldrin.

Reproduction in *Trichoderma* spp. is through light induced asexual conidia (Papavizas, 1985). Concentric rings of conidial formation are observed in culture in response to alternating light-dark conditions. Exposure to as little as 3 min of daylight is sufficient for some strains to sporulate. Conidial germination requires external nutrients. Therefore, to ensure establishment and proliferation in the soil, biocontrol preparations usually contain a nutrient base.

Table 1.1. Taxonomic scheme outlined by Gams and Bissett (1996).

Section	Type species	Examples	Morphological Features
Trichoderma	*T. viride*	*T. atroviride, T. harzianum*[a], *T. koningii, T. lignorum*	Colony growth - slow to fast, reverse colourless to dull yellowish, some isolates produce distinct coconut odour Conidiation occuring in loose tufts or compact pustules - white at first turning green (rarely brown) Conidia green
Pachybasium	*T. hamatum*[b]	*T. crassum, T. longipile, T. strictipile, T. tomentosum, T. virens*	Colony growth - slow to fast, reverse colourless to yellow, amber or reddish Conidiation occuring in loose tufts or compact pustules - white to grey, green or brown Conidia colourless to grey, green or brown
Longibrachiatum	*T. longibrachiatum*	*T. citinoviride, T. reesei T. pseudokoningii*	Colony growth - rapid, reverse in fresh isolates conspicuously yellowish-green Conidiation occuring in loose tufts, green Conidia green
Hypocreanum	*T. lacteum*	*Hypocrea* anamorphs (rarely seen)	Examples in this section have been discovered mainly through culturing of ascospores of species of *Hypocrea* - the anamorphs are rarely seen independently of the teleomorphs, and as such membership in this section can be viewed as a functional grouping rather than being indicative of a natural relationship

[a] *T. harzianum* has recently been included in the section *Pachybasium*

[b] *T. hamatum* has been re-classed in the *Hypocrea rufa* complex (Sarah Dodd, pers. comm.)

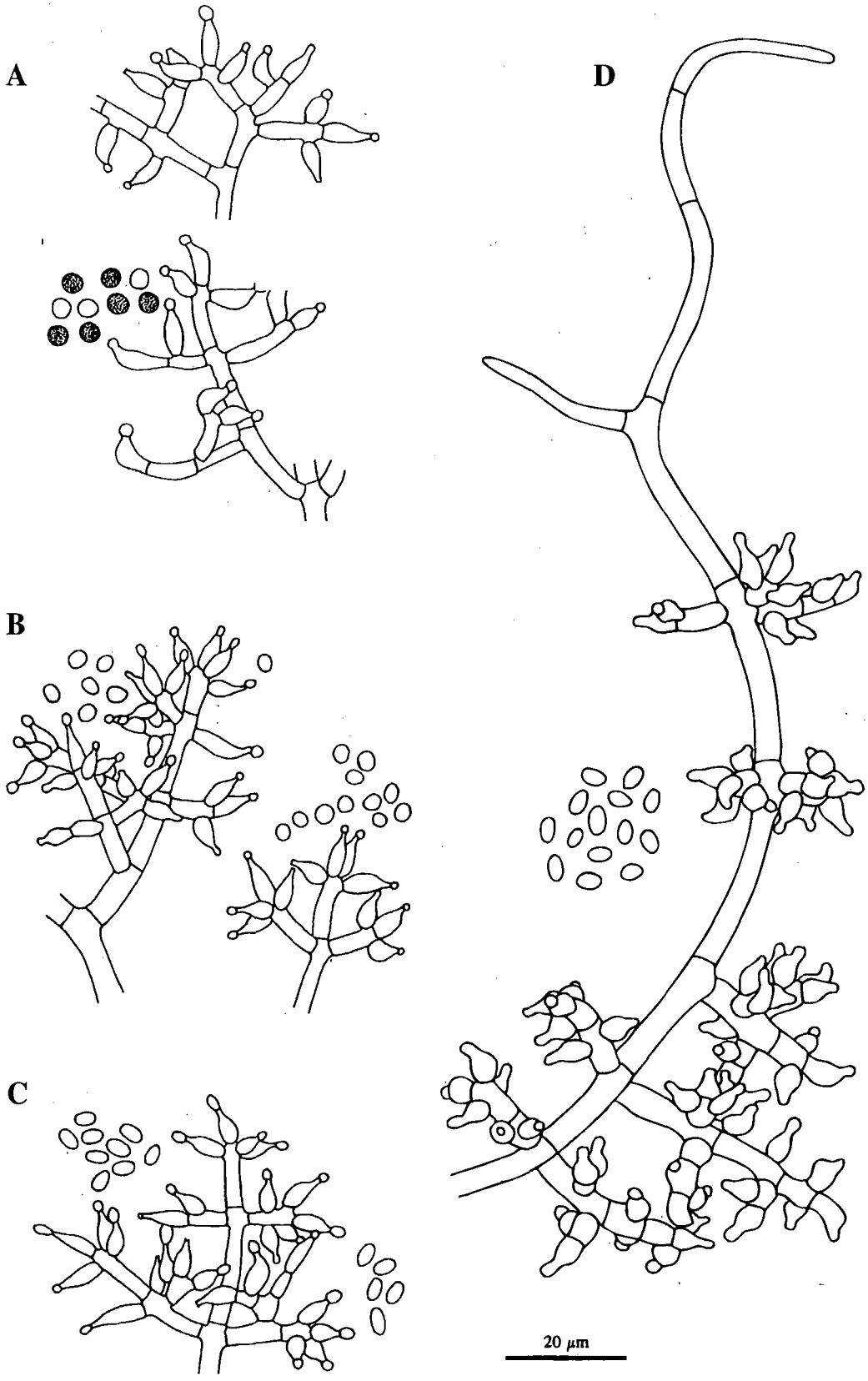


Figure 1.2. A-C *Trichoderma* section *Trichoderma*. **A.** *T. atroviride*, **B.** *T. harzianum*, **C.** *T. koningii*. **D.** *Trichoderma* section *Pachybasium*: *T. hamatum*, from Gams and Bissett (1998).

To be an effective biocontrol agent of root disease, a microorganism needs to multiply and proliferate in the rhizosphere, however *Trichoderma* spp. are not naturally rhizosphere competent (Ahmed and Baker, 1987). Rhizosphere competent mutants have been genetically created, the first instance of which was accidental. Ahmed and Baker (1987), in creating a benomyl-resistant *T. harzianum*, discovered their mutant to also be rhizosphere competent.

Many of the qualities that make *Trichoderma* superior saprophytes also made them attractive 'work-horses' for humans. *Trichoderma* produces cellulases for the metabolism of cellulosic substances (Samuels, 1996). Industry has exploited these cellulases in a range of areas, including laundry detergent and the conversion of sugar by-products to protein for animal feed. Cellulases, chitinases and other hydrolytic enzymes are thought to be responsible for the degradation of fungal cell walls, termed mycoparasitism. Mycoparasitism has been exploited on a large scale as a means of biocontrol of soilborne fungal phytopathogens.

1.2.3 Biological Control of Plant Pathogens

Effective biological control of fungal phytopathogens by *Trichoderma* is usually achieved through a combination of competition, antibiosis and mycoparasitism (Hjeljord and Tronsmo, 1998). Competition occur when two or more microorganisms demand more of a resource than is locally available. As a highly efficient saprophyte, *Trichoderma* can rapidly utilise available nutrients at the expense of other soil inhabitants.

Antibiosis was defined by Cook and Baker (1983) as the inhibition or destruction of one organism through the action of metabolites produced by another. Various *Trichoderma* isolates produce secondary metabolites of antifungal and/or antibacterial nature, and while they are not the major mechanism of biocontrol, they play an important role in combination with other factors (Howell, 1998). Antibiotics isolated from *Trichoderma* include 6-pentyl-α-pyrone (PAP), and peptaibols. These have been isolated from *T. polysporum*, *T. koningii* and *T. atroviride*.

1.3 Mycoparasitism: A Biocontrol Mechanism

Mycoparasitism of fungal phytopathogens by *Trichoderma* has been considered its main mechanism of biological control (Chet *et al.*, 1998). It involves the direct attack of one fungus on another, and in the case of *Trichoderma* generally involves the production of cell wall lytic enzymes.

1.3.1 Steps in Mycoparasitism

Mycoparasitism involves four sequential steps: chemotropism, recognition, attachment and coiling, cell wall lysis (Chet *et al.*, 1998). Chemotropism is the directed growth of an antagonist towards its host, usually along a chemical gradient of amino acids and/or sugars and is not considered essential to mycoparasitism. Recognition is essential as many antagonists, including *Trichoderma,* are host specific. In *Trichoderma*, recognition is believed to involve binding of host lectins to carbohydrate residues on the *Trichoderma* cell wall (Inbar and Chet, 1994). Immediately following recognition, *Trichoderma* hyphae attach to and coil around the host hyphae by the formation of hook- and appressorium-like structures, an example of which is provide in Figure 1.3.

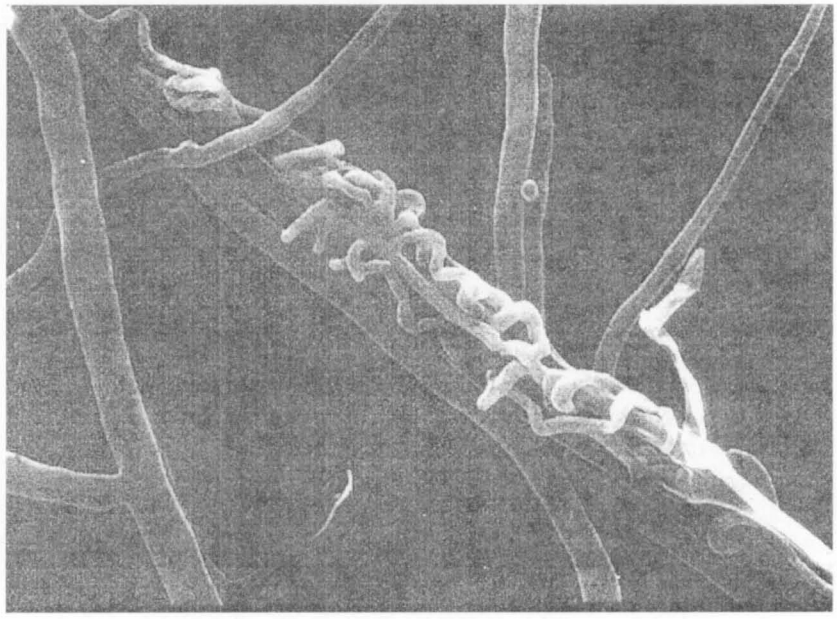


Figure 1.3. Scanning electron micrograph of *Trichoderma* hyphae coiled around *R. solani* hyphae (Elad *et al.* (1983) in Chet *et al.*, 1998).

The penultimate step in mycoparasitism is cell wall lysis. This is accomplished by the action of chitinases, glucanases (including cellulases) and proteinases (Chet *et al.*, 1998) and may also involve the action of *Trichoderma* produced antibiotics (Schirmböck *et al.*, 1994). Penetration of the cell wall has often been observed, and parasitising hyphae visualised growing inside the host hyphae.

Lysis of fungal cell walls by mycoparasitic strains of *Trichoderma* involves degradation of the major cell wall components (Haran *et al.*, 1996a). The fungal cell wall is mainly composed of

polysaccharides (80%) and proteins (3-20%), with lipids, pigments and inorganic salts present in lower amounts (Ruiz-Herrera, 1992). The main macromolecular components in higher fungi are β-glucan, chitin and mannoproteins (glycoproteins), in the lower fungi cellulose predominates over chitin. β-glucan, chitin (chitosan in some fungi) and cellulose microfibrils form the scaffolding responsible for the strength and shape of the wall (Bartnicki-Garcia, 1968; Ruiz-Herrera, 1992; Haran *et al.*, 1996a; Kubicek and Penttilä, 1998) . Mannoproteins are a component of the matricular material in which the scaffolding is imbedded. Also included in the matricular material are the lipids, inorganic salts and pigments. Cell wall degrading enzymes (CWDEs) have been categorised on the basis of their substrates (Table. 1.2) (Haran *et al.*, 1996a; Benítez *et al.*, 1998; Koivula *et al.*, 1998; Lorito, 1998).

Table 1.2. Summary of cell wall lytic enzymes identified in *Trichoderma* spp. and their substrates .

Group Name	Specific Names	Features
β-glucanases	Exo- β-1,3- or β-1,6-glucanases	Cleave β linkages from the non-reducing ends of the chain
	Endo-β-1,3- or β-1,6-glucanases	Cleave β linkages internally at random in β-glucans and their oligomers
	β-1,3- or β-1,6-glucosidases	Cleave oligo- and disaccharides
Cellulases	β-1,4-D-glucan cellobiohydrolases exocellulases	Cleave cellobiose units from the ends of cellulose and its oligomers
	Endo-β-1,4-glucanases	Cleave β linkages internally at random
	β-1,4-glucosidases	Cleave cellobiose units to glucose
Chitinases	Chitin β-1,4-chitibiosidase exochitinases	Cleaves β linkages from the non-reducing end of chitin and its oligomers
	Endochitinase	Cleave β linkages internally at random in chitin and its oligomers
	β-1,4-N-acetylhexosaminidase exochitinases	Cleave chitin, its oligomers and chitobiose from the non-reducing end
Proteinases		Attack specific amino acid residues within the polypeptide chain

1.4 Role of Cell Wall Degrading Enzymes in Mycoparasitism

1.4.1 Evidence from Biological Studies of *Trichoderma* species

β-1,3-glucanases, chitinases and cellulases have been implicated as the mechanism of biocontrol action by *Trichoderma*. Chet and Baker (1981) isolated a *Trichoderma* sp., identified as *T. hamatum*, from a *R. solani* suppressive soil in Columbia, South America. In dual plate assays, they observed typical mycoparasitic action: overgrowth of the *R. solani* by *Trichoderma*, coiling and hyphal degradation at the site of interaction. When the *Trichoderma*

was grown on a medium containing *R. solani* cell walls, β-1,3-glucanases and chitinases were released into the medium. Addition of the *Trichoderma* to soil resulted in significant disease reduction of *R. solani* on beans. By inference from the *in vitro* assay, β-1,3-glucanases and chitinases were implicated as the mechanism of disease reduction. The same *Trichoderma* was able to grow on cellulose as the only carbon source, and soil incorporation resulted in significant disease reduction of *Pythium* spp. on peas. Together these findings implicated cellulase(s) as the mechanism of action, as *Pythium* spp. are Oomycetes (cellulose instead of chitin in the cell wall) (Table. 2.2.2).

Parasitism of persistent survival structures such as sclerotia is an important biocontrol feature. β-1,3-glucanases and chitinases have been implicated in sclerotial parasitism by *Trichoderma.* Elad *et al.* (1984) looked at three *Trichoderma* isolates identified as *T. harzianum.* All three could parasitise *R. solani* but only one was able to attack the *S. rolfsii* sclerotia. β-1,3-glucanases and chitinases were isolated from the soil and in sclerotia. SEM studies on the sclerotia revealed they had lost their regular shape and holes had appeared.

A 1993 study by Lorito *et al.* studied the antifungal effects of purified enzymes in order to implicate a role in biocontrol activity. They used the *Trichoderma* strain P1, initially identified as a *T. harzianum* later re-identified as a *T. atroviride.* They isolated and purified chitobiosidase(s) and endochitinase(s) and supplied it in potato-dextrose broth to the chitin-containing fungi *Fusarium solani, Botrytis cinerea,* and *Ustilago avenae*, and to the cellulose-containing Oomycete *Pythium ultimum.* Strong inhibition of growth and development was observed with all fungi except *P. ultimum.* The authors cited this study as evidence for the biocontrol activity of chitinolytic enzymes.

More direct evidence of lytic enzyme involvement in mycoparasitism came from SEM studies of the distribution of gold-labelled GlcNAc in a mycoparasitised *F. oxysporum.* Using a *Trichoderma* sp. isolated from commercial peat, Chérif and Benhamou (1990) set up dual cultures with the *Fusarium.* Two days after inoculation, the part of the *Fusarium* colony facing the *Trichoderma* showed marked alterations in cell wall labelling. At these sites considerable amounts of gold particles accumulated , however, there was no ultrastructure modification. After four days, partial to complete wall disintegration and release of the cytoplasm contents was observed, however no fungal penetration occurred. This study demonstrated that cell wall lysis was due in part to the action of *Trichoderma* chitinases.

1.4.2 Evidence from Genetic Studies of *Trichoderma* species

The advent of molecular cloning techniques in the late 1980s has facilitated the study of *Trichoderma* enzymes at the molecular level. Examples of each enzyme type listed in Table 1.2 have been isolated from *Trichoderma* and some of these enzymes have been cloned and sequenced (Table 1.3) ((Benítez *et al.*, 1998; Kubicek and Penttilä, 1998; Lorito, 1998; and references therein) Cohen-Kupiec *et al.*, 1999; Donzelli *et al.*, 2001).

Table 1.3. Cell wall degrading enzymes isolated from *Trichoderma* spp. and implicated in biocontrol activity.

Enzyme		Gene (if cloned)
β-glucanases	Exo-β-1,3-glucanase (110 kDa)	*lam1.3*
	Exo-β-1,3-glucanase (40 kDa)	-
	Exo-β-1,3-glucanase (31 kDa)	-
	Exo-β-1,3/β-1,4-glucanase (70 kDa)	-
	Endo-β-1,3-glucanase (78 kDa)	*bgn13.1*
	Endo-β-1,3-glucanase (70 kDa)	-
	Endo-β-1,3-glucanase (36 kDa)	-
	Endo-β-1,6-glucanase (51 kDa)	-
	Endo-β-1,6-glucanase (43 kDa)	*bgn16.1*
	β-1,3-glucosidase (78 kDa)	*gluc78*
Cellulases	Cellobiohydrolase I	*cbh1*
	Cellobiohydrolase II	*cbh2*
	Endo-β-1,4-glucanase I	*egl1*
	Endo-β-1,4-glucanase II	*egl2*
	Endo-β-1,4-glucanase III	*egl3*
	Endo-β-1,4-glucanase IV	*egl4*
	Endo-β-1,4-glucanase V	*egl5*
	β-glucosidase I	*bgl1*
Chitinases	Chitobiosidase - CHIT40[a]	-
	Endochitinase - CHIT52	-
	Endochitinase - CHIT42	*chit42 (ThEn-42, ech-42, chi1)*
	Endochitinase - CHIT37	-
	Endochitinase - CHIT33	*chit33*
	Endochitinase - CHIT31	-
	β-N-acetylhexosaminidase - CHIT102	-
	β-N-acetylhexosaminidase - CHIT72 (CHIT73)	*chit72 (nag1, exc1)*
	β-N-acetylhexosaminidase - CHIT64	-
	β-N-acetylhexosaminidase - CHIT28	-
Proteinases	Serine Protease	*prb1*

[a]Chitinase names are based on apparent molecular weight on SDS-PAGE. It is likely that cloning and sequencing will reveal some of the isolated enzymes to be the same product.

Carsolio *et al.* (1994) demonstrated increased expression of a *Trichoderma* endochitinase during mycoparasitism of *R. solani*. *Ech-42* mRNA rose in the presence of *R. solani* on confrontation plates when the mycelium were in direct physical contact implicating a role in

mycoparasitism. Flores *et al.* (1997) demonstrated increased biocontrol activity by integrating multiple copies of the *prb1* proteinase gene into the genome of *T. atroviride*. They obtained a 5-fold increase in disease protection compared to the wild type. However, while all transformants showed greater biocontrol activity than the wild type, the transformant with intermediate production (P2) afforded the greatest disease protection. All transformants had a reduced growth rate, with P2 being the least affected and it is possible that other proteins important in biocontrol were degraded by the high protease activity.

No difference in *in vitro* biocontrol activity was observed between *T. atroviride chit42* overexpression mutants, deletion mutants and wild type (Carsolio *et al.*, 1999). The ability of *chit42* deletion mutants to offer the same level of protection supported the view that combined action of multiple enzymes is required for biocontrol activity. Loss of one enzyme did not affect the outcome and illustrate the complexity of the mycoparasitic response.

1.5 Genetic Regulation of the Mycoparasitic Response

The complex mechanisms involved in the regulation of mycoparasitic genes in *Trichoderma* have not been fully elucidated. From the literature, regulation appears to involve an inducer/repressor system. The exact nature of the inducer *in vivo* has not been determined, however a catabolite repressor protein has been suggested to be involved. Also, the association of genes into apparent 'complexes' and their coordinated expression has raised the possibility of regulatory cascades.

1.5.1 The Nature of the Host-Derived Inducer

Mycoparasitic induction can occur prior to physical contact with a host, suggesting an inducer molecule diffusing from the host to the *Trichoderma* mycelium (Córtes *et al.*, 1998). Growth of *Trichoderma* on host cell walls results in induction of the mycoparasitic lytic enzymes, however the exact nature of the host-derived inducer molecule(s) has still not been determined. Several putative inducers have been identified by their *in vitro* induction capabilities, but whether they act as the major inducer(s) *in vivo* remains to be shown. More recently the existence of a global inducer, which may switch on a generalised mycoparasitic response has been suggested (Córtes *et al.*, 1998).

β-1,3-glucanase inducers

β-1,3-glucanases are semi-constitutive enzymes inducible by several polysaccharides including laminarin, starch, xylose, mannitol and glycerol (Sivan and Chet, 1989). The levels of production vary depending on the carbon source, in particular the number of β-1,3-glucan linkages (Vázquez-Garcidueñas *et al.*, 1998). In addition, different enzyme profiles are induced in relation to carbon source. As β-1,3-glucanase production is semi-constitutive, it is possible that the enzyme is constitutively released into the environment where it liberates glucan monomers from appropriate hosts, which then diffuse back to *Trichoderma* and induce expression via a positive feedback mechanism.

Cellulase inducers

Abundant cellulase production has been observed when *Trichoderma* spp. are grown on cellulose or mixtures of plant polymers, however, as cellulose cannot enter the cell, it is believed that cellulose oligomers may be the inducer (Kubicek and Penttilä, 1998). Previous work has demonstrated constitutive conidial-bound cellulases to be involved in cellulase induction by cellulose (El Gogary *et al.*(1989) in Seiboth *et al.*, 1992). The predominant cellobiohydrolase in conidia is CBH II and in mycelium CBH I. Seiboth *et al.* (1992) created *cbh2* over-expression and deletion mutants to examine its role in induction. Over-expression of *cbh2* resulted in more efficient induction of cellulases by cellulose. In the deletion mutant, there was a considerable lag in growth and cellulase induction when grown on cellulose.

Chitinase inducers

Chitinases have been induced *in vitro* by chitin, chitin degradation products, and various sugars including xylose, lactose and mannose (Sivan and Chet, 1989; Ulhoa and Peberdy, 1991; Ulhoa and Peberdy, 1993; Córtes *et al.*, 1998; Schickler *et al.*, 1998; Mach *et al.*, 1999). Chitin, like cellulose, is unable to enter the cell however, basal amounts of chitinases can be detected under non-inducing condition and, like β-1,3-glucanase and cellulase, it is postulated this basal level may be enough to initiate degradation of host chitin, thus releasing degradation products which induce chitinase expression via positive feedback mechanisms (Ulhoa and Peberdy, 1991; Inbar and Chet, 1995; Schickler *et al.*, 1998).

Proteinase induction and the possibility of a global inducer

Trichoderma atroviride proteinase (*prb1*) expression has been induced by chitin, a non-substrate of the enzyme, but not by the addition of proteins such as bovine serum albumin and

casein (Geremia *et al.*, 1993). The induction of *prb1* by chitin, which also induces chitinase expression, raises the possibility of a common induction pathway for hydrolytic enzymes. If such a global inducer of the mycoparasitic response does exist it is unlikely to be chitin, as *prb1* expression has also been induced on *T. viride* cell membranes (no chitin present), and mycoparasitism of cellulose-containing Oomycetes has been well documented.

The possibility of a host-derived global inducer has been suggested by Córtes *et al.* (1998). In a confrontation assay where *R. solani* and *T. atroviride* were separated by cellophane, *chit42* and *prb1* were both strongly induced, which indicated a diffusible molecule from the host to be the inducer. An autoclaved soluble extract from *R. solani* cell walls filtered to <30 kDa also induced *chit42* and *prb1* strongly. Treatment of the extract to remove proteins, glycoproteins and lipids did not reduce induction, therefore they postulated the inducer to be a polysaccharide. When this work was repeated using dialysis membrane in place of cellophane, no *chit42* induction was observed (Zeilinger *et al.*, 1999). This discrepancy was investigated by Kullnig *et al.* (2000), who noted that dialysis allowed diffusion of molecules up to 12 kDa, whereas cellophane allowed up to 90 kDa. Using both cellophane and dialysis in a confrontation assay with *T. atroviride* versus *R. solani* they demonstrated that a molecule of 12-90 kDa in size must diffuse from *Trichoderma* to the host prior to induction of *chit42*. A molecule >12 kDa in size then diffuses to *Trichoderma* triggering *chit42* expression.

Whilst the exact nature of the diffusible molecule, and possible global inducer, has not been determined, its potential has already being exploited (Kubicek *et al.*, 2001). Possible inducers from *R. solani* cell walls have been tested for effects *in vitro* on biocontrol activity and enzyme production, with the intent of commercial exploitation.

1.5.2 The Role of Lectin Recognition in Induction

In the absence of a diffusible medium, host cell wall lectins may serve as the mycoparasitic response inducer. Host cell wall lectin has served as a recognition signal which induces coiling and appressorium-like formation in *Trichoderma* (Inbar and Chet, 1994). In a biomimetic system using purified *S. rolfsii* cell wall lectin bound to nylon fibres, lectin induced production of *T. harzianum* CHIT102 (Inbar and Chet, 1995). In temporal expression studies CHIT102 is the first chitinolytic enzyme to be induced (Haran *et al.*, 1996b), thus in addition to triggering mycoparasitic structure formation, lectin recognition may also trigger induction of chitinolytic enzymes needed for host cell wall penetration.

Using the same lectin-coated biomimetic system, Córtes *et al.* (1998) observed no induction of *chit42* and *prb1*, and thus concluded that lectin recognition was not sufficient to induce the complete response. However it is important to note that Córtes *et al.* (1998) used a *T. atroviride* isolate, whereas the biomimetic studies above involved *T. harzianum.* The biomimetic system used in both studies contained no chitin. In the *in vivo* situation, in the absence of a diffusible medium, it is possible that induction of other chitinase genes may require chitin breakdown products produced through the action of CHIT 102.

Induction of mycoparasitism through lectin-carbohydrate binding may be transduced from the host cell-surface by heterotrimeric G proteins and mediated by intracellular cAMP. Using the G protein activator mastoparan Omero *et al.* (1999) were able to induce mycoparasitic-like structures indistinguishable from those observed in the biomimetic system in both *T. harzianum* and *T. atroviride.* Significant differences were observed between species, with *T. atroviride* showing the greatest response Addition of cAMP, a likely downstream target of G protein activation, also resulted in the formation of mycoparasitic structures.

1.6 Transcription Factors Implicated In Regulation

Examination of the promoter regions of the mycoparasitic lytic genes has suggested possible mechanisms of regulation. Chitinase induction was inhibited by RNA and protein synthesis inhibiters, which indicated that *de novo* transcription and translation are necessary for production (Ulhoa and Peberdy, 1991). A global inducer of mycoparasitism would, therefore, interact directly or affect an internal mycoparasitic response protein, which in turn interacts with the genes at the transcriptional level. If common regulatory pathways do exist, conserved motifs should be present within the promoter regions of mycoparasitic genes. Conserved regions have been found, implicating, among other things, catabolite repression and stress induction to be possible modes of regulation.

Carbon catabolite repression

Carbon catabolite repression is considered a major regulatory mechanism in both prokaryotes and eukaryotes (Ilmén *et al.*, 1996). It involves binding of specific proteins to promoter sequences in response to carbon nutrient conditions. In high glucose conditions, glucose is metabolised preferentially through the repression of structural genes required for utilisation of other carbon sources. Catabolite repression of the lactose operon in *E. coli* (Watson *et al.*,

1987) and the ethanol and proline operons in *Aspergillus nidulans* (Kulmburg *et al.* (1993) in Ilmén *et al.*, 1996) have been well documented.

Repression of mycoparasitic genes in the presence of glucose implicated catabolite repression to be involved in negative regulation. Only basal levels of β-1,3-glucanases were detected when grown in glucose as the sole carbon source, and production under inducing conditions was inhibited by the addition of glucose (Vázquez-Garcidueñas *et al.*, 1998). Cellulase production was repressed in the presence of glucose and derepressed when glucose in the medium is exhausted (Kubicek and Penttilä, 1998). Endochitinase production was reduced to basal levels in the presence of glucose (Margolles-Clark *et al.*, 1996), however N-acetylhexosaminidase production was not, indicating it to play a role in cellular functions other than mycoparasitism (Ulhoa and Peberdy, 1993). Proteinase (*prb1*) production was also repressed in the presence of glucose (Geremia *et al.*, 1993).

A *Trichoderma* catabolite repressor gene (*cre1*) has been cloned, and molecular studies have confirmed its role in cellulase repression (Ilmén *et al.*, 1996). A *cre1*$^-$ mutant strain produced high levels of *cbh1* when grown on glucose, whereas *cbh1* was not detected in the wild type. Interestingly, expression studies in *T. reesei* showed that *cre1* mRNA was low when glucose was the sole carbon source and higher on neutral carbon sources such as cellulose. This was unexpected and did not fit the model of CRE1 as a catabolite repressor. This may suggest that CRE1 has roles additional to catabolite repression as has been suggested for *A. nidulans* CREA.

Sequence analysis has revealed *cre1* DNA binding motifs in the promoters of *chit42*, *prb1* (Córtes *et al.*,1998) *chit33* (de las Mercedes *et al.*, 2001) *nag1* (Mach *et al.*, 1999), *gluc78* (Donzelli *et al.*, 2001), *cbh1* & *cbh2* (Kubicek & Penttilä, 1998), further implicating catabolite repression in regulation of mycoparasitism. Using two fragments from the 5' non-coding region of *T. atroviride chit42*, each containing multiple *cre1* consensus binding sites (5'-SYGGRG-3'), Lorito *et al.* (1996) examined the relative protein binding abilities in the non-mycoparasitic and mycoparasitic state. In the non-mycoparasitic state, the DNA fragments bound to CRE1, and in the mycoparasitic state they bound to an unknown protein, a putative mycoparasitic response regulator. Based on these results they formed a putative model for regulation of *chit42* expression during mycoparasitism (Figure 1.4).

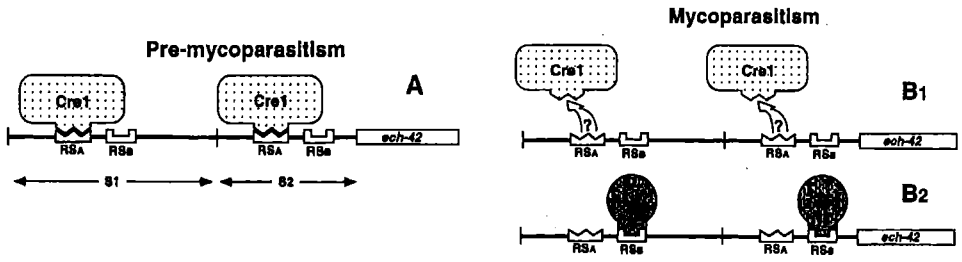


Figure 1.4. Putative model for regulation of *chit42* (*ech42*) gene expression during mycoparasitism. RSa: Cre1 binding sequences. RSb: mycoparasitic regulator binding sequences. From Lorito *et al.* (1996).

When protein extracts from both states were mixed, CRE1 competitively bound to the fragments, suggesting that Cre1 became functionally impaired during transition to mycoparasitism. *In vitro* studies have implicated phosphorylation to be the mechanism of impairment (R.L Mach & C.P Kubicek (unpublished) in Kubicek-Pranz, 1998).

Stress response element binding sites

A putative stress response element binding site, CCCCT (C_4T), has been found in the promoter regions of *chit42*, *prb1* (Córtes *et al.*, 1998) and *gluc78* (encodes a 78 kDa β-1,3-glucosidase) (Donzelli *et al.*, 2001) indicating that response to stress is a possible regulatory mechanism. In *Saccharomyces cerevisiae*, C_4T is bound by the zinc finger transcription factors Msn2p and Msn4p (Kobayashi & McEntee, 1993). Mach *et al.* (1999) observed that *chit42* was induced by ethanol, low temperatures, high osmotic pressure and during carbon starvation. Recently, orthologues of *msn2/msn4* have been cloned from *T. atroviride* and the encoded protein demonstrated to bind to C_4T elements (Peterbauer *et al.*, unpublished in Kubicek *et al.* (2001)).

BrlA and AbaA binding sites

Two BrlA binding boxes (5' MRAGGGR-3') were found in the *T. atroviride chit42* promoter region, implicating induction during light-induced sporulation (Carsolio *et al.*, 1994). BrlA is a regulatory protein that modulates expression of several light-induced genes involved in sporulation in *A. nidulans* (Chang & Timberlake, 1992) and may also play a role in gene induction during carbon starvation (Skromne *et al.*, 1995). Carsolio *et al.* (1994) found a higher level of *chit42* expression in *Trichoderma* cultures grown in the light versus the dark.

Two AbaA binding motifs (5'-CATTCY-3') have been identified in the promoter region of *T. atroviride gluc78*. Like BrlA, AbaA is involved in regulating conidiation in *A. nidulans* (Andrianopoulos & Timberlake, 1994).

GATA sites

Seven putative GATA sites in the *prb1* and two in the *chit42* are present in the promoter region of *T. atroviride* (Córtes *et al.*, 1998). GATA factors are a class of eukaryotic transcriptional regulators that recognise a six base pair sequence with a GATA core, (consensus 5'HGATAR-3') (Scazzocchio, 2000). These sequences are recognised in *A. nidulans* and *S. cerevisiae* by positive regulators of major physiological processes including nitrogen metabolism, siderophore biosynthesis and the blue light response (Conion *et al.*, 2001). Their role in *Trichoderma* is unknown.

The role of AceI and AceII binding sites in cellulase induction

Using a novel yeast-based system, two transcription factors, AceI and AceII, were isolated from *T. reesei* and their associated DNA binding motifs were identified in the promoter regions of *cbh1* and *cbh2* (Saloheimo *et al.*, 2000; Aro *et al.*, 2001). Disruption of either *ace1* or *ace2* resulted in reduced growth on cellulose-containing medium, and reduced mRNA expression of both *cbh1* and *cbh2*. The authors of these studies believed AceI and AceII may act cooperatively in the regulation of cellulase expression. Interestingly the AceII recognition site was identical to the binding site determined for XlnR, a regulator of *A. niger* hemicellulases and cellulases.

CCAAT binding factors and putative binding sites for unknown proteins

CCAAT sequences have been found in the promoters of the majority of eukaryotic genes, and bind a variety of different transcription factors. To date only one binding complex (HAP) has been identified in fungi (Brakhage *et al.*, 1999). The HAP-complex consists of at least four subunits, which bind cooperatively to CCAAT sequences interacting with other regulatory proteins by creating an open chromatin structure (Narendja *et al.*, 1999). In fungi, the HAP-complex is required for activation of gene expression by pathway specific regulators and plays a major role in the control of genes involved in respiration. HAP-complexes are important in regulation of *cbh2* from *T. reesei* (Zeilinger *et al.*, 1998; Zeilinger *et al.*, 2001). Genes encoding *hap 2/3/5* orthologues have been cloned and sequenced from *T.* reesei and a HAP complex consisting of at least HAP 2/3/5 was shown to bind to the CCAAT-box.

Gene induction of *T. atroviride nag1* by chitin monomers involved binding of an unknown protein to the sequence 5'-CCAGN_{13}CTGG-3' within the promoter region. The nature of this protein is not known, however a CCAAT-box located within the spacer region may have coordinated binding via a HAP complex.

The identity of the mycoparasitic response regulator

Putative mycoparasitic response regulator binding sites have been identified in the promoter regions of *T. atroviride chit42* and *prb1* (Córtes *et al.*, 1998). Comparison of the *chit42* and *prb1* promoter regions revealed four identical sequences in both promoters (MYC1 - MYC4), located at similar positions relative to the transcription start point (Figure 1.5). Transgenic *Trichoderma* strains lacking the *chit42* MYC4 sequence had reduced *chit42* expression, which suggested this motif was necessary for full expression of the chitinase gene. In addition, MYC3 in *chit42* overlapped a CRE1 binding motif by two base pairs, supporting the model of regulation proposed by Lorito *et al.* (1996) (Figure 1.4).

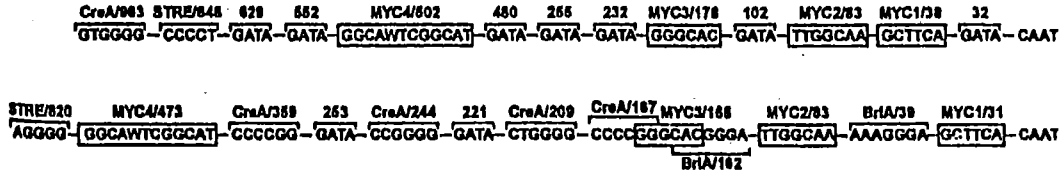


Figure 1.5. *prb1* and *chit42* promoter regions. The numbers indicate the position relative to the CAAT box. From Córtes *et al.* (1998).

1.7 Organisation of Genes Into Complexes

A large number of mycoparasitic hydrolytic enzymes have been found in *Trichoderma* spp. (Table 1.4), many of which are grouped within *Trichoderma* isolates, suggesting organisation of genes into complexes. Vázquez-Garcidueñas *et al.* (1998) found seven different β-1,3-glucanases in *T. harzianum* and Haran *et al.* (1995) found six chitinolytic enzymes present in *T. harzianum*. Various studies have demonstrated temporal expression of these genes. Inbar and Chet (1995) examined the activity of *T. harzianum* CHIT 102 and CHIT 73 in dual cultures with *S. rolfsii*. Prior to contact, small constitutive amounts of CHIT 102 were detected, and 12 hours after contact the levels increased. At 24 hours, CHIT 102 began to disappear and CHIT 73 was detected, and from then until the end of the incubation period,

CHIT 73 was the major chitinolytic enzyme. Córtes *et al.* (1998) found that on a chitin supplemented medium *T. atroviride prb1* expression reached a maximum at 72 h, whereas *chit42* reached a maximum at 110 h. They postulated that the difference reflects coordinated regulation where Prb1 was required to release the inducer which then triggered chitinase expression. In addition, Carsolio *et al.* (1994) have demonstrated that *chit42* maps to the same chromosomal band as *prb1*. Differential expression of genes within a complex may extend host range.

1.8 Synergism in the Mycoparasitic Response

Effective biocontrol activity of *Trichoderma* spp. *in vivo* requires the synergistic action of hydrolytic enzymes and antibiotics. Concentrations of individual hydrolytic enzymes produced *in vivo* were far below concentrations required for effective fungicidal activity *in vitro*, however combinations of enzymes with different modes of action substantially increased activity *in vitro* (Ghisalberti *et al.* (1990) in Schirmböck *et al.*, 1994). In studies using deletion mutants, loss of one hydrolytic enzyme had no apparent effect on mycoparasitic activity, indicating biocontrol activity to be a function of combinations of enzymes (Carsolio *et al.*, 1999). Schirmböck investigated whether synergism also existed between *T. atroviride* antibiotics and hydrolytic enzymes. Both peptaibols and hydrolytic enzyme synthesis was induced in the presence of *B. cinerea* cell walls, and a synergistic effect on *B. cinerea* and *F. oxysporum* hyphal elongation and spore germination was observed when purified antibiotics and enzymes were added together versus singularly.

Synergism has also been demonstrated between commercial fungicides and *Trichoderma* hydrolytic enzymes. Lorito *et al.* (1994) investigated synergism between β-1,3-glucosidase, endochitinase, a β-N-acetylhexosaminidase and five different antifungal compounds: gliotoxin, flusilazole (a triazole), miconazole (an imidazole), benomyl and captan. Every combination showed a substantial level of synergism in the inhibition of *B. cinerea* spore germination. The greatest synergism was with antifungals that act on cellular membranes, gliotoxin, flusilazole and miconazole, suggesting that combined attack on the cell wall and membrane may be particularly damaging for the host. This finding demonstrated excellent potential for use of *Trichoderma* spp. as part of an IPM programme using reduced levels of fungicide.

1.9 Background To Research

Five *Trichoderma* isolates were isolated from local soils and screened for biocontrol activity against *Sclerotinia sclerotiorum* and *S. minor* as part of a Ph.D. thesis submitted by Nimal Rabeendran (Rabeendran, 2000). Of these five, four were identified by Gary Samuels[1] as *T. longipile* and one as *T. tomentosum.* Three of the *T. longipile* isolates were chosen as the subjects of this study. A summary of the biocontrol results is presented in Table 1.4. The main features were a reduction in inoculum potential and disease producing activities of the pathogens.

Table 1.4. Biocontrol activity of three *Trichoderma* isolates against *Sclerotinia* diseases in a range of laboratory and field trials.

Isolate	Cabbage Petiole assay (*S. sclerotiorum*)	Cabbage Pot Assay (*S. sclerotiorum*)	Cabbage Field Trial (*S. sclerotiorum*)	Lettuce Field Trial (*S. minor*)
6Sr4	reduced infection and inhibition of sclerotial formation	no reduction in apothecial production	reduced disease	reduced disease
S1BYG	reduced infection and inhibition of sclerotial formation	reduced apothecial production	not included in trial	reduced disease
3Sr4-2	reduced infection and inhibition of sclerotial formation	reduced apothecial production	not included in trial	no disease reduction

Mycoparasitism by 6Sr4, S1BYG and 3Sr4-2 towards fungal phytopathogens was observed in dual culture plate assays (McLean, 2002, pers. comm.). 6Sr4 demonstrated mycelial parasitism of *Aphanomyces euteiches* (an Oomycete), *B. cinerea* (higher fungi), and all isolates demonstrated parasitism of *S. sclerotiorum* (Figure 1.6).

The mechanism of the biocontrol activity of these isolates against *Sclerotinia* diseases in the field has not been determined, however based on the *in vitro* plate assays and literature pertaining to other members of the *Trichoderma* genera, mycoparasitism was likely to be a key mechanism.

[1a] Gary Samuels (USDA, Beltsville, MD, USA) is recognised as a world expert in *Trichoderma* taxonomy

Figure 1.6. Mycoparasitism of 6Sr4 towards *Aphanomyces euteiches.* Note the peg-like structures and hyphal penetration

1.10 Summary and Objectives

The natural ability of *Trichoderma* to parasitise other fungi as a source of carbon has led to their exploitation as biocontrol agents of soilborne fungal diseases, the full potential of which has yet to be realised. Inconsistencies in disease reducing abilities in the field have hampered wide-spread implementation of *Trichoderma* species as key players in IPM programmes. This has been due to a lack of full understanding of the regulatory mechanisms underlying mycoparasitism, and failure to consider *Trichoderma* in an ecological context. Cell wall degrading enzymes (CWDEs) have been implicated as the major factors responsible for mycoparasitism. Characterisation of the genes encoding CWDEs and identification of key regulatory triggers will allow for greater uniformity of biocontrol activity through manipulation of environmental parameters. Misidentification of isolates has hampered interpretation of regulatory studies to date. In addition, the large numbers of enzymes differentially induced within each complex and their associated multiple cis and trans acting factors involved in regulation have slowed progress in understanding the fundamentals of mycoparasitism.

Using the *Trichoderma* isolates described in section 1.9 as model systems the objectives of this study were to:

- Establish identity of the isolates through molecular techniques.

Analysis of gene regulation in *Trichoderma* has been seriously hampered by misidentification. Ribosomal gene sequence analysis has been demonstrated as a reliable method of taxonomic identification. The first objective of this thesis is to unambiguously identify 6Sr4, S1BYG and 3Sr4-2 using this method.

- Isolate three putative mycoparasitic genes for further study

A wealth of biological, biochemical and molecular studies have implicated cell wall degrading enzymes as playing key roles in mycoparasitism. It is likely these enzymes are involved in mycoparasitism by all biocontrol *Trichoderma* species. The second objective of this thesis is to isolate and sequence orthologues of genes implicated in mycoparasitism from 6Sr4, S1BYG and 3Sr4-2.

- Identify potential binding motifs through characterisation of the regulatory region

Analysis of the regulatory regions of mycoparasitic genes has implicated several factors that are likely to be involved in gene induction, including Cre1, Msn2p/Msn4p and BrlA. In addition, sequence alignment between different genes has revealed putative binding sites for global inducers of mycoparasitism. Conservation of binding motifs between species may idenifiy active binding sites. The third objective of this thesis is to analyse the regulatory regions of genes implicated in mycoparasitism for the presence of binding motifs and putative global inducer motifs.

- Investigate gene expression under inducing conditions

Induction of genes implicated in mycoparasitism has been demonstrated in response to a wide variety of substrates. The fourth objective of this study is to compare gene expression patterns of the isolated genes and to establish a role for their enzymes in mycoparasitism.

Potential outcomes:

- Increased understanding of the biocontrol activity of *Trichoderma*

Substantial data is required on the genotype/phenotype of a singular species, the knowledge of which can then be applied to other *Trichoderma.*

- Identification of positive inducers for incorporation into inoculation amendments

Variability of biocontrol activity in the field can be attributed to many factors, however local environmental parameters may be controlled through inoculation amendments. Incorporation of specific mycoparasitic inducers into inoculation formulations may optimise and standardise biocontrol activity in the field.

Chapter Two
Taxonomy

2.1 Introduction

The traditional system of fungal classification is based largely on sexual reproduction strategies, however, the majority of economically important species lack an observable sexual stage. Identification of imperfect fungi relies heavily on subjective analysis of microscopic asexual features, often leading to inaccuracies in the literature. The advent of molecular technologies has provided taxonomists with more reliable and objective methods for identification, with ITS sequence analysis the most commonly applied method.

Neo-Darwinism is a modern theory which links genetic variation within a specific DNA sequence to evolutionary relatedness. Slowly evolving sequences, often those encoding for essential cellular functions, are useful for studying distantly related organisms, whereas faster evolving sequences allow for comparative study of more closely related individuals. The DNA region which encodes the ribosomal subunits (rDNA) is under high functional constraint, contains both slow- and fast-evolving sequences and is present in all cell types, thus making it ideal for phylogenetic examinations as well as species determination. The rDNA sequences of eukaryotic cells are transcribed as a single rRNA precursor molecule (Watson *et al*, 1987). Separating the ribosomal genes are the non-coding transcribed spacer regions (ETS and ITS), which are spliced during RNA processing (Figure 2.1). As ETS and ITS are non-coding, they are under low functional constraint and therefore evolve more rapidly than the ribosomal subunits, proving useful for analysis at the genus and/or species level (White *et al*, 1990).

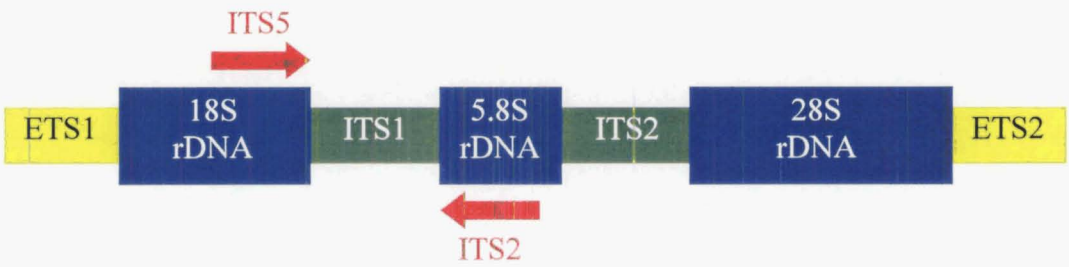


Figure 2.1. Nuclear rRNA precursor molecule. ETS = External Transcribed Spacer, ITS = Internal Transcribed Spacer. Primers ITS5 and ITS2 are used to amplify the ITS1 region.

Comparative analysis of the ITS regions of *Trichoderma* species has proven to be the most reliable method for identification (Samuels, 1996; Kindermann *et al*, 1998). The *Trichoderma* isolates in this study were identified as *T. longipile* on the basis of morphological characters, by Dr Gary Samuels (USDA, Beltsville, MD), who is recognised as a world leader in *Trichoderma* taxonomy (Alison Stewart, pers. comm.). Morphology has proven on many occasions to be difficult for accurate identification of *Trichoderma* species, therefore it is possible the identification of these isolates as *T. longipile* may not be correct. To verify the identity of the isolates used in this study ITS1 sequence analysis was performed.

2.2 Materials and Methods

2.2.1 Isolates

Three New Zealand-sourced *Trichoderma* isolates (6Sr4, S1BYG and 3Sr4-2) were used in this study (Rabeendran, 2000). All three had been previously identified as *T. longipile* on the basis of morphological characters. In addition JD2, a United Kingdom *T. hamatum* isolate, was included in this analysis (Dodd *et al*, 2000). All isolates were maintained and stored on potato-dextrose agar (PDA) as described in Appendix 7.2.1.

2.2.2 DNA Extraction

Genomic DNA was isolated from all *Trichoderma* isolates (2.2.1) using the Genomag™ kit (Advanced Biotechnologies Ltd., Surrey, UK) essentially as per manufacturer's instructions. Approximately 500 mg of frozen mycelium (Appendix 7.2.2) was placed in a sterile mortar and pestle containing liquid nitrogen. The mycelium was ground to a fine powder and 100 mg transferred to a cooled (liquid nitrogen) centrifuge tube. The ground mycelium was immediately homogenised in 500 µL of dilute Prep Buffer II (cell lysis), using a wide-bore pipette tip, and placed on ice. Samples were incubated at 65°C for 30 min, with inversion of tubes every 5 min. Tubes were cooled, 120 µL of Prep Buffer IV added and placed on ice for 15 min. Solution was centrifuged at 20 000 x*g* for 5 min at 4°C, and the supernatant containing DNA transferred to a fresh tube. DNA was precipitated by addition of one volume of 80% isopropanol, incubation at -20°C for 1 h, and pelleted by centrifugation at 20 000 x*g* for 15 min at 4°C. The pellet was washed twice in 70% ethanol, air-dried, resuspended in 100 µL of sterile water and stored at 4° C. DNA samples were quantified via gel electrophoresis as described in Appendix 7.2.3.

2.2.3 Amplification of the ITS1 Region

Amplification of the ITS1 region was performed using the previously described primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2 (5'-GCTGCGTTCTTCA TCGATGC-3') (White *et al*, 1990). Each 25 µL amplification reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP (Advanced Biotechnologies Ltd.), 10 pmoles of each primer, 10 ng of *Trichoderma* DNA and 1.25 U Taq DNA polymerase (Roche Molecular Biochemicals Ltd. Mannheim, Germany). Controls containing all of the above ingredients except genomic DNA were included. Amplification was performed in an Eppendorf Mastercycler® Gradient PCR machine (Eppendorf-Netherler-Hinz GmbH, Hamburg, Germany) and consisted of an initial denaturation of 2 min at 94° C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72C°, and a final extension of 7 min at 72°. The resulting PCR products were visualised by 1% agarose gel electrophoresis as described in Appendix 7.2.3.

2.2.4 ITS1 Sequence Analysis

The PCR products obtained above were sent to the Waikato DNA Sequencing Facility (The University of Waikato, Hamilton) for sequencing in both directions using the ITS2 and ITS5 primers at concentrations of 5 pmoles μL^{-1}.

Identity to other ITS1 regions was analysed using GenBank's Blastn function (www.ncbi.nlm.nih.gov). Homologous sequences or those with high similarity were aligned and homology trees constructed using DNAman™ (Version 2.6, Lynnon Biosoft, Quebec, Canada).

2.2.5 Characterisation of Colony and Hyphal Morphology

To examine colony morphology, a mycelial plug from stored slope cultures was transferred to PDA (Beckton Dickson & Co.) in Petri dishes (approximately 25 mL) and grown for 2 d at 20°C under 12 h light/dark cycles. Isolates were sub-cultured using a 6 mm diameter cork borer to thinly-poured PDA plates (~15 ml), and grown for 2-3 weeks at the above temperature to allow for conidiation. To examine hyphal and conidial morphology, isolates were also sub-cultured to sterile glass slides, which were placed in Petri dishes containing moist filter-paper. These humid chambers were incubated as above for 1 week, the plug was then removed and observations made under a light microscope at 100X and 400X magnification. The mycelium was also mounted in water and viewed under the compound microscope.

2.3 Results

2.3.1 ITS1 Sequence Analysis

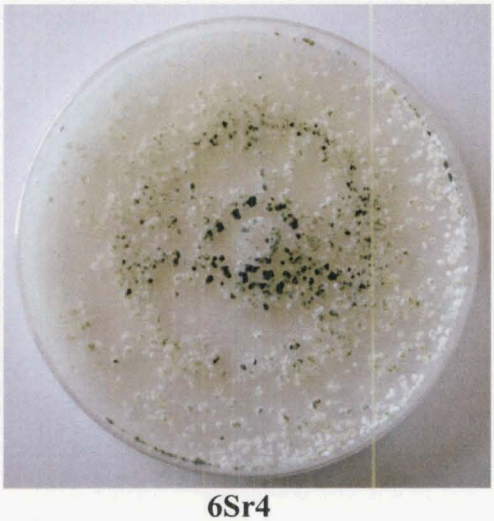
A single 300 bp band at a concentration of approximately 30 ng μL^{-1} was amplified from all *Trichoderma* isolates. The ITS1 DNA sequences of 6Sr4, S1BYG and 3Sr4-2 were identical. When aligned, these sequences shared 81% identity with *T. longipile* ITS1 sequences deposited in GenBank (ascn#: AF011985 and AF011975) (Figure 2.2). A blastn search in GenBank with the 6Sr4 sequence and subsequent alignments revealed 100% identity to *T. hamatum* (ascn#: Z48816). The ITS1 region of *T. hamatum* isolate JD was also sequenced and had 100% identity to 6Sr4, S1BYG and 3Sr4-2.

Analysis of the sequence alignments revealed a one nucleotide addition at position 81 in isolates 6Sr4, S1BYG, 3Sr4-2 and JD2 when compared to the majority of *T. hamatum* sequences deposited in GenBank. Two independent ITS1 sequences for *T. hamatum* neotype DAOM 167057 were deposited in GenBank, one had the nucleotide addition at position 81 (ascn#: Z48816), the other did not (ascn#: AF011957).

Figure 2.2. Homology tree based on ITS1 sequence alignment. 167057 = DAOM 167057, the neotype for *Trichoderma hamatum*. Homology tree was constructed in DNAman™ (Lynnon Biosoft).

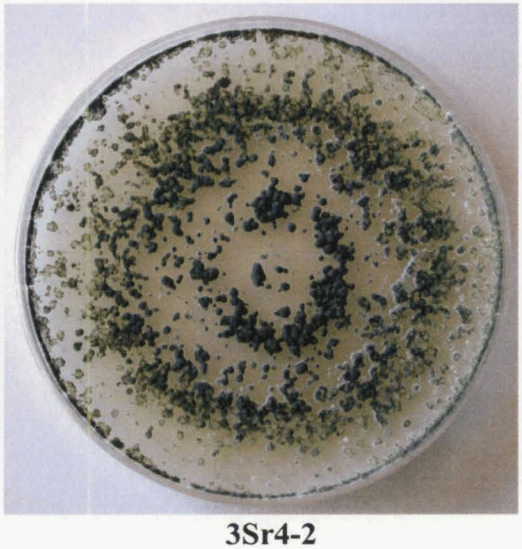
2.3.2 Morphological Description of 6Sr4, S1BYG and 3Sr4-2

Each isolate displayed a characteristic colony morphology when grown on PDA. 6Sr4 was distinct from S1BYG and 3Sr4-2 in conidial pigmentation and rate of differentiation (Figure 2.3). S1BYG formed initials in a defined ring on day 2 and bluish-green conidia on day 4. 3Sr4-2 formed initials in a less defined ring on day 3 and bluish-green conidia on day 4. 6Sr4 developed initials on day 4 and white conidia in a near-random pattern on day 5. Green-yellow conidial pigmentation did not occur in 6Sr4 until day 10. In S1BYG and 3Sr4-2, conidial differentiation occurred in concentric circles, a 12 h light effect, these circles were only faintly visible in 6Sr4. At day 25, the conidial pigmentation of 6Sr4 more closely resembled that of 3Sr4-2 (Figure 2.3)



6Sr4

S1BYG

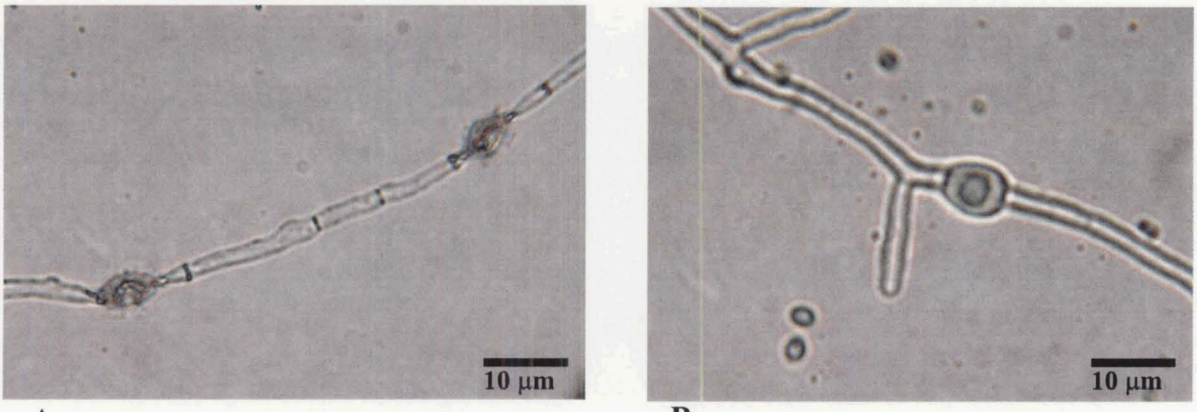


3Sr4-2

Figure 2.3. 10 day old *Trichoderma* cultures on PDA.

S1BYG and 3Sr4-2 shared typical *T. hamatum* colony characteristics (Bissett, 1991). Conidiation was in concentric circles, at first white then bluish-green. The colony morphology of 6Sr4 was not typical of either *T. hamatum* or *T. longipile*. Scattered white pustules appeared, white at first, slowly changing to green-yellow conidia.

Microscopic examination of 6Sr4, S1BYG and 3Sr4-2 hyphae revealed a high level of branching and extensive chlamydospore differentiation (Figure 2.4). These features are characteristic of *T. hamatum* and absent in *T. longipile*.



A B

Figure 2.4. 6Sr4 mycelium showing chlamydospores. **A.** Dry-slide. **B.** Mounted in water.

2.4 DISCUSSION

ITS1 sequence analysis and morphological observations indicated that the three *Trichoderma* isolates 6Sr4, S1BYG and 3Sr4-2, which were originally identified on the basis of morphological characters as *T. longipile*, were *T. hamatum*.

The homology tree indicated 100% conservation with *T. hamatum* sequences deposited in GenBank, however the actual sequence alignments revealed a one nucleotide difference at position 81 of the alignment. Two independently derived ITS1 sequences of the *T. hamatum* neotype DAOM 167057 were present in GenBank. One of these sequences had the same nucleotide addition, the other did not. Assuming both sequences are correct, this difference may reflect normal variation within the population, indicating that a one base pair difference is acceptable in defining this species through ITS1 sequence analysis. Indeed, it has been observed that different cultures of the same isolate may vary by one base-pair within the ITS

region (Sarah Dodd, pers. comm.). However, small amounts of variation within the ITS1 region are considered sufficient for species definition, for example only a three base pair difference separated *T. hamatum* (ascn#: Z48816) from *T. asperellum* (ascn#: AF149863).

The four *T. hamatum* (*longipile*) isolates were originally identified on the basis of morphological characters as defined by Bissett (1991). According to this definition the majority of morphological characters defining *T. hamatum* and *T. longipile* overlap. The major distinguishing features were the lack of observed chlamydospores in *T. longipile*, and highly branching conidiophores in *T. hamatum* versus minimal branching in *T. longipile* except at the apex. Re-evaluation of the hyphal morphology showed all isolates had the defining characters of *T. hamatum*. In the original identification, colonies were grown on cornmeal agar. The possibility exists that hyphal morphology may have varied on the different media.

Although the ITS1 regions of 6Sr4, S1BYG and 3Sr4-2 were identical, their relative colony morphologies were quite distinct, and these differences were also apparent soon after isolation (Rabeendran, 2000). All cultures were isolated from Canterbury soils, S1BYG and 3Sr4-2 from intensive commercial horticultural soils in Marshlands, North Canterbury, and 6Sr4 from a field site at Lincoln University which was cropped in potato and bean. Soil types in these two areas were very different, the former being considerably higher in organic matter, and this may have had an effect on colony morphology.

Whilst *T. hamatum* and *T. longipile* are morphologically similar, ITS1 analysis showed they were more distinct than morphology would indicate. Both species share the same ecological niche, that is temperate soils, however so do many other *Trichoderma* species, such as *T. harzianum*, which is morphologically as well as genetically distinct from both *T. hamatum* and *T. longipile*. It is more likely the similarities are indicative of the inherent difficulties in *Trichoderma* taxonomy. *Trichoderma* species are mainly asexual, proven teleomorphs (*Hypocrea* species) being few. A review by Samuels (1996) states the asexual characters used to differentiate species are not variable enough, or are difficult to describe in *Trichoderma*.

From the data presented here it would appear that neither morphological nor genetic characters alone are sufficient for accurate identification of *Trichoderma hamatum*, rather a combination of morphological and molecular taxonomic techniques were required.

Chapter Three
Regulation of Genes Implicated in Mycoparasitism

3.1 Introduction

Analysis of gene structure facilitates predictions about gene regulation and function. For example, analysis of the promoter regions of *Trichoderma* mycoparasitic genes has revealed common consensus regulatory motifs, such as the Cre1 binding site(s) (Ilmén *et al.*, 1996), which are involved in gene regulation.

One of the methods previously employed to isolate *Trichoderma* mycoparasitic genes is targeted PCR (Fekete *et al.*, 1996). Targeted PCR involves the design of oligonucleotide primers based on aligned DNA sequences from a number of organisms for a particular gene. These primers can then be used to amplify similar gene(s) from the target organism. Once a portion of the gene has been sequenced, a technique called inverse PCR can be used to derive the full gene sequence, including the upstream regulatory region.

Traditionally, gene cloning involves using gene fragments or orthologues to probe a cDNA or genomic DNA library. Depending on gene size it may be possible to recover clones containing the entire gene construct. In addition, complementation studies can provide extensive information on sequences flanking the gene. The major disadvantage is the cost and time involved in generating a library. In addition, if only 2-3 copies of the gene are present in the library, polymorphism within an isolate, such as heterokaryosis, may be difficult to detect. Inverse PCR is more cost effective in comparison, and sequencing of multiple clones allows for detection of heterokaryosis.

Based on observed mycoparasitism in *in vitro* plate assays and literature pertaining to other members of the *Trichoderma* genus, it is likely that the previously observed biocontrol activity of the *T. hamatum* isolates used in this study (Rabeendran, 2000) is due mainly to mycoparasitism. Previous research has established that cell wall hydrolytic enzymes play a pivotal role in mycoparasitism of fungal phytopathogens by biocontrol species of *Trichoderma*, therefore it is likely that these *T. hamatum* isolates also produce such enzymes. The aim of this investigation was to isolate and sequence mycoparasitic gene homologues from the biocontrol agent *T. hamatum* 6Sr4 using targeted and inverse PCR, and partially characterise the promoter regions using sequence and gene expression analysis.

3.2 Materials and Methods

3.2.1 Fungal Isolates and Sequences

All *Trichoderma* isolates used in this study are from New Zealand and were identified by morphology and ITS sequencing (Chapter 2). They included three *Trichoderma hamatum* 6Sr4, S1BYG and 3Sr4-2, and a *T. harzianum* TV#. The soilborne fungal pathogen *Sclerotinia sclerotiorum* isolate G1, used in the direct confrontation assay, was from Montana, USA and had been identified on the basis of morphology (Belinda Sleight, pers. comm.). Isolates were maintained and stored on PDA (Appendix 7.2.1). All sequences used for alignments were downloaded from Genbank (www.ncbi.nlm.gov/Genbank) (Table 3.1).

Table 3.1. Origin of sequences from gene implicated in mycoparasitism and cell wall degrading enzymes.

Gene	Isolate	Species	ITS ascn	Ascn
chit42	Tam-61	*T. hamatum*	-	U88560
	P1	*Trichoderma atroviride*	AF278794	Z80358
	IMI206040	*T. atroviride*	AF278795	X79381
	Gv29-8	*T. virens*	-	AF050098
prb1	IMI206040	*T. atroviride*	AF278795	M87518
xbg1.3-110	T-Y	*T. harzianum*	-	AJ002397
exg1	SB111	*Cochliobolus carbonum*	-	L48994

3.2.2 Extraction of Genomic DNA for use in PCR Amplifications

Genomic DNA (gDNA) was isolated from frozen mycelium using the Genomag™ kit (Advanced Biotechnologies Ltd.) (Section 2.2.2). DNA prepared for use in inverse PCR was precipitated with 2 volumes of 100% ethanol rather than isopropanol, as this can inhibit restriction digestion (Sambrook *et al.*, 1989). Purified DNA was resuspended in 100 µL of sterile water and stored at 4°C. DNA samples were quantified via gel electrophoresis as described in Appendix 7.2.3.

3.2.3 Isolation of *chit42* (*ThEn-42, ech-42, chi1*)

3.2.3.1 Specific Primer Design

Using the published sequence of *T. hamatum chit42* (ascn#: U88560), two primer sets were designed to amplify the *chit42* gene from 6Sr4. Primers ECH-315 (5'-TGCTTGC TGCGCTGCAGG-3') and ECH-1612 (5'-AGACCTTGGTGTTGATCAATG-3') were designed to exon 1 and 4, respectively, and were predicted to amplify a 1.3 kb region. Primers ThamECH-1 (5'-ATCGATCGGTCTGGCATTAT-3') and ThamECH-1590 (5'-TTAGTGAAGTAGACAGCGTT-3') were designed to amplify a predicted 1.6 kb region of the *chit42* promoter.

3.2.3.2 Amplification of *chit42*

Using primers designed above, putative *chit42* gene sequences were amplified from *Trichoderma hamatum* 6Sr4. All PCR amplifications were performed in either an Eppendorf Mastercycler® Gradient PCR machine (Eppendorf-Netherler-Hinz GmbH) or a Perkin Elmer Model 2400 Thermal Cycler (Perkin Elmer Cetus Corp., Norwalk, Connecticut, USA). Each 25 µL PCR reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP (Roche Molecular Biochemicals Ltd.), 10 pmoles of each primer, 10 ng of *T. hamatum* DNA and 1.25 U *Taq* DNA polymerase (Roche Molecular Biochemicals Ltd.). DNA from *T. harzianum* TV# and *T. koningii* MTM# was amplified as positive controls. To check for contamination, a negative control was included that contained all the above ingredients except DNA. Single primer controls containing all the above ingredients and only one of the two primers were also included, to assess whether one primer could amplify in both directions.

Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. For primer set ThamECH-1/ThamECH-1590 an annealing temperature of 55°C was used. Resulting PCR products were separated by 1% agarose gel electrophoresis as described in Appendix 7.2.3.

Where more than one band was present, a further aliquot of the PCR product was separated by 2% agarose gel electrophoresis (Appendix 7.2.3), as this increased separation of small bands. A band of the expected size was excised with a 2-200 µL sterile pipette tip and used as a template in a further PCR reaction using the conditions outlined above (but excluding gDNA). The resulting amplimer was visualised by 1% agarose gel electrophoresis.

3.2.3.3 TA Cloning of PCR Products

PCR products obtained above were ligated into the multi-cloning site in pGEM®-T (Promega Corp., Madison WI, USA) (Appendix 7.4) as per manufacturer's instructions and transformed into *E. coli* strain INVαF' (Invitrogen Corp.) using standard techniques (Sambrook *et al.*, 1989). Typically, a 2 µL aliquot of the ligation was mixed with 50 µL of competent cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 30 s to facilitate DNA uptake, then placed on ice for 2 min. A 250 µL aliquot of SOC medium (Appendix 7.2.4) was added and transformations incubated at 37°C for 1 h to allow ampicillin resistance gene expression. A 40 µL aliquot of the transformation mix was spread to LB/Amp/X-Gal selection plates (Appendix 7.2.4) and incubated overnight at 37°C. Clones containing the inserted PCR product were selected on the basis of white colony formation. All clones were maintained on LB/Amp agar and stored in 40% glycerol at -80°C as described in Appendix 7.2.5.

Plasmid DNA from five clones per transformation was prepared using the Perfectprep® Plasmid Mini kit (Eppendorf-Netheler-Hinz GmbH) as per manufacturer's instructions. To confirm the vector contained the appropriate insert, 2 µL of a 1/200 dilution of purified plasmid DNA was amplified using ECH-315/ECH-1612 as described above. PCR products were separated by 1% agarose gel electrophoresis (Appendix 7.2.3) alongside the original PCR product used in the ligation. Two clones per transformation were sequenced at the Waikato DNA Sequencing Facility (University of Waikato).

3.2.3.4 DNA Sequencing of Cloned Inserts

Sequencing of the putative *chit42* gene fragments was performed using both the T7 and SP6 promoters flanking the multi-cloning site in the pGEM®-T vector (Promega Corp.) (Appendix 7.4). Due to the large size of the amplimers cloned above, 1.3 and 1.6 kb, the T7 and SP6 sequences did not overlap. Additional primers were designed to allow sequencing of the remaining nucleotides. ECH-462 (5'-GTACGGTTGTGTC AAGCAACTG-3') was used to sequence the coding region clones and ECH-539 (5'GAAACCTGGCAGCGTCC-3') was used to sequence the promoter region clones. To establish identity of the DNA inserts, the primer sequences were removed and the complete sequence subjected to a Blastn search in GenBank (www.ncbi.nlm.gov/Genbank).

3.2.4 Isolation of *prb1*

3.2.4.1 Amplification, Cloning and Sequencing of the *prb1* Coding Region

A primer set had been designed previously within our laboratory to amplify a 1.1 kb region of the *prb1* gene from *T. atroviride* (Sarah Dodd, pers. comm.). The upstream primer (PRB1-F35) was designed to the signal peptide coding region (5'-AGCTTTGCTCCCGGCTGTCC-3'). The downstream primer (PRB1-R1212) was designed to exon 2 (5'-CCAACAACGTGAGGTGTAGCCATG-3'). These primers were used to amplify the *T. hamatum prb1* gene from 6Sr4. Amplifications were performed at an annealing temperature of 55°C and resulting PCR products cloned and sequenced as described in Section 3.2.3.

3.2.4.2 Derivation of the Upstream Regulatory Region of *prb1*

The *prb1* coding region sequence derived above was analysed using DNAman™ (version 2.6, Lynnon Biosoft) for non-cutting 6 bp restriction enzyme recognition sequences. A 500 ng aliquot (Caroline Young, pers. com.) of 6Sr4 gDNA was digested to completion using *Hind*III, according to manufacturer's instructions, in a total volume of 100 µL. Digestion was confirmed by 1% agarose gel electrophoresis of a 10 µL aliquot alongside 50 ng of uncut DNA. The restriction enzyme was removed by phenol/chloroform extraction (Appendix 7.2.2) and the digested DNA reprecipitated using 1/10 volume 3 M sodium acetate and 2 volumes 100% ethanol, followed by incubation on ice for 15 min. DNA was collected by centrifugation at 14 000 x*g* at 4°C, the pellet washed three times in 70% ethanol, air dried and re-dissolved in 100 µL sterile distilled water.

Digested DNA (200 ng) was circularised by ligation with 1 U T4 DNA ligase (Roche Molecular Biochemicals Ltd.), according to manufacturer's instructions, in a total volume of 100 µL. The ligase was removed by phenol/chloroform extraction (Appendix 7.2.2), the DNA reprecipitated (as described above) and re-dissolved in 20 µL sterile distilled water. All DNA was stored at -20°C.

Inverse primers I-PRB-509 (5'-GCTTCTTGTGAGGGCTGC-3') and I-PRB-1067 (5'-GGTGTTAACATTCTGTCGTC-3') were designed to a region approximately 50 bp inside the known 6Sr4 *prb1* DNA sequence. PCR reaction components were essentially as described in section 3.2.3.2, except that 50 ng of circularised restriction-digested DNA was used as a template instead of 10 ng of gDNA. Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C and 2

min at 72°C, followed by final extension of 10 min at 72°C. A gDNA control (10 ng) was included to assess possible amplification of any undigested DNA. Inverse-PCR products were separated by 1% agarose gel electrophoresis as described in Appendix 7.2.3.

Amplification of the *Hind*III prepared template yielded a 2 kb inverse product, which was enriched by excision from the 1% agarose gel and reamplification using reaction times and temperatures as described above. Amplification was confirmed through 1% agarose gel electrophoresis.

The *Hind*III inverse PCR product was ligated to pGEM®-T (Promega Corp.) and transformed into the *E. coli* strain INVαF' (Invitrogen Corp.) as described in section 3.2.3.3. Circularisation of the restriction fragments should have reconstructed the restriction site, and this was confirmed by digesting 10 µL of the amplified insert with *Hind*III according to manufacturer's instructions. Restriction patterns were examined by 1% agarose gel electrophoresis. Plasmid DNA from clones containing a single restriction site were sequenced as described in section 3.2.3.4. An additional primer, IPRBint500 (5'-CAACTCGGATGTCGATATGG-3') was used to sequence an internal 200 bp region. Sequences were compiled to produce a complete sequence of the *prb1* gene from 6Sr4 including the coding and promoter regions.

3.2.5 Isolation of *xbg1.3-110* (*lam1.3*)

3.2.5.1 Gene Isolation using Degenerate Primers

Degenerate primers were designed to regions of identity in the protein sequences of the *T. harzianum* (ascn#: #AJ002397) and *Cochliobolus carbonum* (ascn#: L48994) exo-β-1,3-glucanases and predicted to amplify a 485 bp region of the *T. hamatum xbg1.3-110* gene. In order to select a suitable region for degenerate primer design, *T. hamatum* codon preference was determined (Appendix 7.3) through analysis (DNAman™, version 2.6, Lynnon Biosoft) of exon 5 of the *T. hamatum chit42* gene (ascn#: U88560). Two criteria were used to select appropriate regions from the aligned protein sequences. First, they must contain two or more of the amino acids His, Cys, Lys, Glu, Met and Trp which exhibit little or no codon degeneracy. Second, the nucleotide sequence encoding one of these amino acids must appear at the 3' end of the primer. Primer set XBGp394/XBGp535 was designed to the selected regions and where codon preference was less than 100%, degenerate nucleotides were used (Figure 3.1).

Using the primer set XBGp394/XBGp535, an ~500 bp portion of the *xbg1.3-110* gene was amplified from *T. hamatum* 6Sr4, essentially as described in section 3.2.3.2, except 25 pmoles of each primer and an annealing temperature of 46°C were used. The *xbg1.3-110* 485 bp fragment was sequenced in the T7 direction only.

T. harzianum	[392] ThrArgSerLysProGlnTyrGluThrLeuPro [402]
Cochliobolus	SerLysSerLysProGlnTyrGluThrLeuThr
XBGp394	5′ WSHaagccHcaRtaYgag 3′
T. harzianum	[534] MetTrpAspValHisThrArgIleGlyGlyPhe [544]
Cochliobolus	LeuTrpAspValHisThrArgIleGlyGlyAla
nucleotide (sense)	5′ tgggaYgtYcaYacHMgM 3′
XBGp535 (antisense)	3′ accctRcaRgtRtgDKcK 5′

Figure 3.1. *xbg1.3-110* degenerate primer design. Aligned protein sequences are *exg1* from *Cochliobolus* and *lam1.3* from *T. harzianum*. Blue represents regions of 100% homology. Numbering relative to the start codon at 1. Degenerate primers are in red. Letters in lowercase represent single nucleotides. Uppercase letters represent multiple nucleotides: W = a/t, S = c/g, H = a/c/t, R = a/g, Y = c/t, K = g/t, D = a/g/t, M = a/c.

3.2.5.2 Derivation of Full Gene Sequence using Inverse and Degenerate PCR

The predicted *xbg1.3-110* gene was large, therefore a combination of PCR techniques was required to amplify the flanking sequences. The positions of the primers used to amplify *xbg1.3-110* are shown in Figure 3.2.

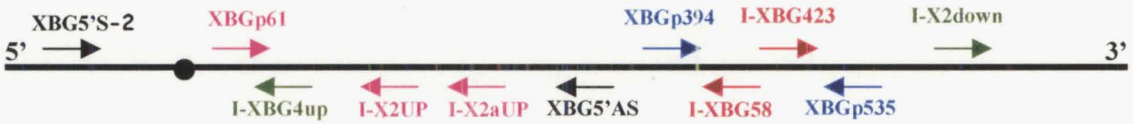


Figure 3.2. Positions of primers used to amplify *xbg1.3-110* from 6Sr4. Black dot represents the ATG start codon. Primers used in sequencing reactions are not shown.

Inverse primers were designed to within 50 bp of the 5' and 3' ends of the known 500 bp (3.2.5.1) and used to extend the *xbg1.1-110* sequence. 6Sr4 gDNA was digested using *Pvu*II and amplified using primers I-XBG-58 (5'-CCTTGACAAACAATGAATAACAG-3') and I-XBG-423 (5'-GTCCTCATCGAGTGGAACTTG-3') as described for *prb1*

(Section 3.2.4.2). The resulting inverse-PCR product (~1.2 kb) was cloned and sequenced, and an extended ~1.7 kb sequence of *xbg1.3-110* was compiled.

PCR with degenerate primers was used to extend the ~1.7 kb sequence. Degenerate primer XBGp61 (5'-aacgTYaaggaYTacggHgcYaag-3') was designed to a region 5' to the 1.7 kb *xbg1.3-110* sequence (Figure 3.3) and used with the sequence specific primer I-X2aUP (5'-TTATTTCGGTACTGCAACGGC-3') to amplify a ~550 bp region upstream of the known sequence. Amplification was performed essentially as described in Section 3.2.3.2, except that 25 pmoles of XBGp61 and an annealing temperature of 56°C were used. Resulting PCR products were separated by 2% agarose gel electrophoresis (Appendix 7.2.3), a band of the expected size (580 bp) excised and then used as a template in a nested PCR reaction with primers XBGp61 and I-XBG2UP (5'-AAGACCAACGTGGTTGTTGC-3') (located 50 bp 5' to I-X2aUP) under the same conditions as above. An internal primer was used to ensure that *xbg1.3-110* was amplified from the original multiband pattern The resulting PCR product was sequenced using the primer I-XBG2up, and the known sequence extended to ~2.2 kb.

Figure 3.3. *xbg1.3-110* degenerate primer design. Aligned protein sequences are *exg1* from *Cochliobolus* and *thb13exog* from *T. harzianum*. Blue represents regions of 100% homology. Numbering relative to the start codon at 1. Degenerate primers are in red. Letters in lowercase represent single nucleotides. Uppercase letters represent multiple nucleotides: Y = c/t, H = a/c/t.

Inverse primer I-XBG4up (5'-TGTTAATTGCTGCCGTGTCGT-3') was designed to within a 50 bp region at the 5' end of the 2.2 kb sequence and, with I-XBG2down (5'-CGTGAATAGAACTGGCTCTG-3'), used to amplify 50 ng of *Nco*I digested and self-ligated template. Amplification conditions were as described in section 3.2.4.2. The resulting inverse-PCR product was cloned and partially sequenced using the T7 and SP6 promoters increasing the known sequence to 3.8 kb. The 5' sequence was extended by sequencing from the clone with primers X5Int-1 (5'-CTGTATAAATGCAACTAGCG-3') and X5Int-2 (5'-TCAGGGCTTTAGAGTGGAGC-3'). All sequences were compiled to

produce a 4 kb partial sequence of the *xbg1.3-110* gene from 6Sr4, which included 1kb of sequence 5' to the ATG start codon.

Sequencing of the degenerate PCR product above (3.2.5.2) resulted in ambiguities, and this most likely was due to heterokaryosis, a common phenomena in *Trichoderma* species (Harman *et al*, 1998). To generate 'clean' sequence information for this region, and confirm the identity of the second inverse PCR product, primers XBG5'S-2 (TGGTTGACAACAGTCATGTACC-3') and XBG5'AS (5'-TGTACTGCTGA TTACCAATGGC-3') were designed to amplify an expected 1.8 kb of the *xbg1.3-110* gene, including 1 kb 5' to the translational start site and encompassing the region amplified by degenerate PCR. PCR reaction components were as described in section 3.2.3.2. Each amplification consisted of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 64°C and 2 min at 72°C, with a final extension of 10 min at 72°C. Resulting PCR products were cloned and sequenced as described in sections 3.2.3.3 and 3.2.3.4. Primers X5Int-1 and XBG4up were used to sequence internal regions of the clone.

3.2.6 Submerged Culture Assay

In *T. harzianum* both *chit42* and *xbg1.3-110* are up-regulated in the presence of their substrates, chitin and laminarin (β1,3-glucan) respectively. To examine whether this was also true for *T. hamatum*, isolate 6Sr4 was grown in high chitin/laminarin, high glycerol, negative glucose submerged culture assay (Córtes *et al.*, 1998) and RNA extracted for northern analysis.

3.2.6.1 Conidial Recovery for use as Inoculum

Conidia from isolates 6Sr4 and *T. harzianum* TV# were required for inoculating submerged culture assays. To minimise variation between assays, conidia were generated in bulk and stored at -80°C in 20% glycerol until used. Briefly, isolates were grown on PDA (Appendix 7.2.1) at 22°C under 12 h light/dark. To each plate, 5 mL sterile H_2O containing Tween 80 (two drops per 500 mL) was pipetted and a glass rod used to scrape the conidia into suspension. Mycelium was removed by filtering the conidial suspension through sterile Miracloth (Calbiochem) and conidia were pelleted by centrifugation at 1000 x*g* for 2 min at 4°C, resuspended in 20% glycerol and stored at -80°C.

3.2.6.2 Experimental Design

For submerged culture assays, a 100 mL flask containing 25 mL of half strength PDB and 2.5 x 10^{-8} conidia, was grown for 14 h at 22°C in constant dark on a rotary shaker (200 rpm). Mycelium was collected by centrifugation at 2000 x*g* for 5 min at 4°C, transferred to minimal media – MM (0.2 g $MgSO_4.7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCl, 1 g NH_4NO_3, 2 mg $FeSO_4.7H_2O$, 2 mg $ZnSO_4.7H_2O$, 2 mg $MnCl_2.7H_2O$ and 2 g asparagine per litre water; pH 5.5) with 0.4% or 2% glycerol and grown for 12 h in the previously described conditions. Mycelium was again collected, and the pellet was separated into five portions - one portion was wrapped in aluminium foil and snap frozen in liquid nitrogen (day 0), and the other four were transferred to MM only, or MM + 2% glycerol with or without 0.75% chitin (practical grade SIGMA: C-7170) (purified according to Skujins *et al.* [1965]). Mycelium was harvested by centrifugation at 24, 48, 72 and 96 h (days 1-4), pressed between Miracloth (Calbiochem) and paper towels to remove excess liquid, wrapped in aluminium foil and snap frozen in liquid nitrogen. The laminarin assay was conducted essentially as for chitin – 14 h 0.5 x PBD, 12 h MM with 0.4% glycerol, except that after the second centrifugation step the pellet was separated into two portions – one portion was snap frozen and the other transferred to 2% glycerol MM with or without 0.5% laminarin (SIGMA: L-9634). Mycelium was harvested at 48 h. All mycelial samples were stored at -80°C until required. Each assay was replicated once.

3.2.7 Direct Confrontation Assay

The orthologues of the three genes isolated in sections 3.2.3 – 3.2.5 were up-regulated during mycoparasitism under negative glucose conditions in *T. atroviride*. To examine whether this was also true for *T. hamatum*, isolate 6Sr4 was grown in a direct confrontation bioassay, and gene expression analysed by northern dot blots.

The direct confrontation assay was done essentially as described by Córtes *et al.* (1998). Cultures were grown on MM (3.2.6) agar, 0.2% glycerol, pH 5.5 for 3 d at 22°C, then 0.5 cm diameter plugs were inoculated (Figure 3.4) to fresh medium of the same, covered in cellophane disks. In pilot studies, fungal growth on the cellophane was poor and this was overcome by lightly sandpapering the cellophane to break surface tension prior to autoclaving in distilled H_2O (Peter Jarvis, pers. com). Treatments consisted of 6Sr4 vs 6Sr4, 6Sr4 vs *Sclerotinia sclerotiorum* G1, and 6Sr4 alone. Mycelium from six plates was combined and harvested by snap freezing at the following time intervals: 38 h post inoculation, 62 h, overlap (~ 86 h) of 6Sr4 and G1 cultures, and full overlap of 6Sr4 and

G1 (~ 110 h). The harvested regions are indicated in Figure 3.4. Corresponding regions were harvested in the other two treatments. All mycelial samples were stored at -80°C until required. The assay was repeated once.

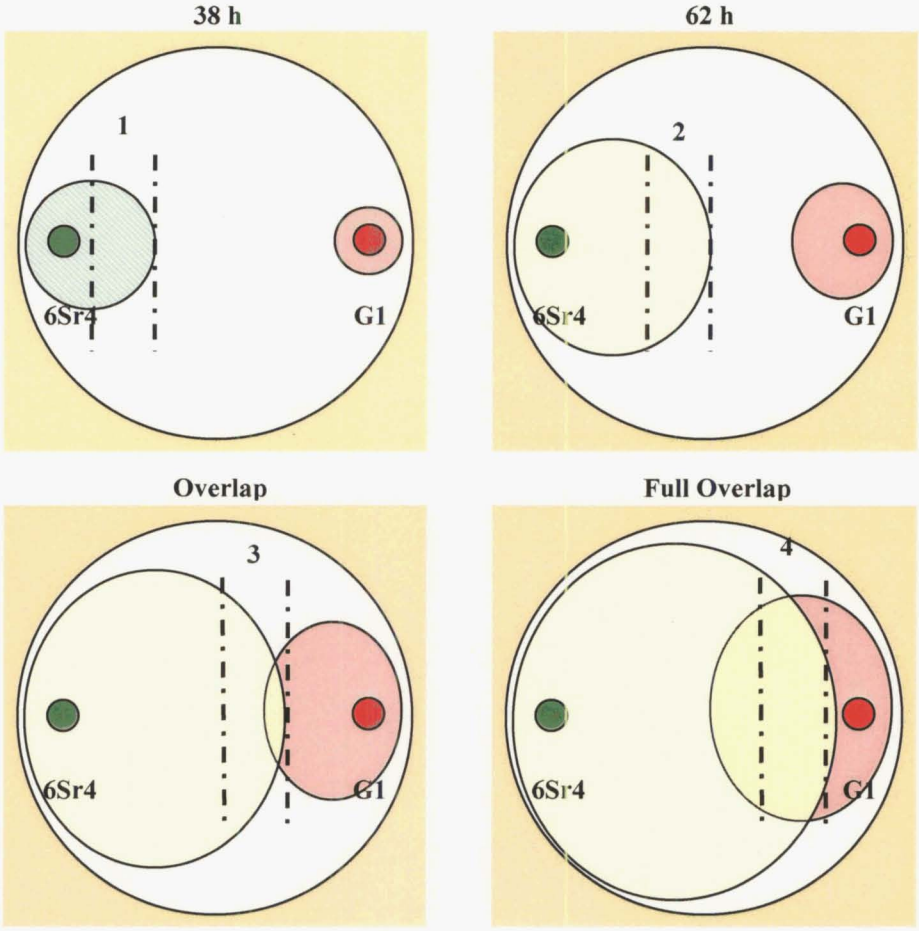


Figure 3.4. Diagrammatic representation of the confrontation assay (6Sr4 vs G1). Mycelium was harvested from within the dashed lines. Green designates 6Sr4 and red designates the G1 colony.

3.2.8 Northern Hybridisation

Northern hybridisation was used to investigate mycoparasitic gene expression of 6Sr4 in the submerged culture and direct confrontation assays (Sections 3.2.6 and 3.2.7).

3.2.8.1 Extraction of RNA from the Submerged Culture and Direct Confrontation assays

Total RNA was extracted from mycelial samples using TRIzol® Reagent (Invitrogen Corp.) essentially as per manufacturer's instructions. Typically, 50 mg of frozen mycelium was placed in a mortar and pestle containing liquid nitrogen, ground to a fine powder and

100 mg transferred to a pre-cooled 1.5 mL centrifuge tube. The ground mycelium was homogenised in 1 mL TRIzol® Reagent and placed on ice until eight samples had been prepared. Samples were incubated for 5 min at room temperature and RNA extracted by the addition of 200 µL chloroform, vigorous shaking for 15 s, incubation at room temperature for a further 3 min, and centrifugation at 20 000 x*g* for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, and RNA precipitated by the addition of 500 µL isopropanol, followed by incubation at room temperature for 10 min. RNA was collected by centrifugation at 12 000 x*g* for 10 min at 4°C. The supernatant was discarded and the pellet washed in 1 mL of 75% ethanol. The pellet was briefly air-dried, and then re-dissolved in 50 mL H_2O. All samples were stored for up to 1 week at -20°C and indefinitely at -80°C. Residual DNA was removed by the addition of 2.5 µL DNAse I (Roche Molecular Biochemicals) and incubation at 37°C for 30 min. DNAse was inactivated by incubation for 5 min at 75°C. RNA concentrations were determined spectrophotometrically as described by Sambrook *et al.* (1989).

3.2.8.2 Probe Generation for Detection of RNA

PCR products amplified from pGEM-T clones containing the appropriate insert were used as probes in this study. PCR products were purified using a PCR-spin column (Bio-Rad Laboratories, Hercules, CA, USA) to remove primers, dNTPs and salts. The DNA was quantified by 1% agarose gel electrophoresis (Appendix 7.2.3) and concentration adjusted to 10 ng μL^{-1}. All probe DNA was stored at -20°C.

The 1.3 and 1.1 kb sections of *chit42* and *prb1* derived in sections 3.2.3 (primer set ECH-315 and ECH-1612) and 3.2.4 (PRB1-F35 and PRB1-R1212) were used as probes for 6Sr4. The corresponding genes in *T. harzianum* TV# were cloned for use as probes using the same conditions described for 6Sr4. A 930 bp insert of *xbg1.3-110* was cloned from 6Sr4 using the specific primers I-Xdirdown1 (5'-GGATCTGCTGGATTCCTCAC-3') and XBG485-428 (5'-GTAGCCAAGTTCCACTCGATG-3').

As a positive control and a measure of total RNA, probes were produced that detected ribosomal RNA (rRNA). A 600 bp region of the rRNA gene cluster was cloned from 6Sr4 and *T. harzianum* TV# using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.*, 1990). The semi-constitutive mitochondrial gene, *cox1*, which encodes a subunit of cytochrome oxidase was also used as a positive control. Whilst rRNA is a good measure of total RNA, mRNA

easily degrades, therefore detection of the *cox1* transcript indicated mRNA stability. A 1.5 kb section of the *cox1* coding region was cloned using primers CBH663 (5'-AGT ACGGCACGGGTTACTG-3') and CBH1405 (5'-TTGATGTTGGAGGAGGTGAC-3), as described in section 3.2.3.2 except the annealing temperature was 55°C.

3.2.8.3 RNA (Northern) Blotting

To prepare the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories), its components were washed in hot water and allowed to air-dry, then UV-irradiated to remove residual RNA. A section of Hybond-N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) at least one well wider than the samples to be blotted was pre-wetted in 5 x SSC (Appendix 7.2.6) and the Bio-Dot was assembled according to manufacturer's instructions. Wells not in use were covered with scotch tape. To each sample well, 100 µL of H_2O was added, and transferred through the membrane by gentle vacuum.

RNA was denatured by dissolving 20 µg of RNA (section 3.2.8.1) in 500 µL of ice-cold 10 mM NaOH, 1 mM EDTA. Samples were applied immediately to the Biodot wells using a gentle vacuum. Individual wells were covered with scotch tape once the solution had drained to avoid damaging the membrane through excessive vacuum pressure. The tape was removed and a further 500 µL of ice-cold denaturing solution applied to wash RNA from the well walls. The tape was removed and maximum vacuum applied until all wells were dry. The Bio-Dot was disassembled and the membrane rinsed briefly in 2 x SSC, 0.1% SDS. Blots were air-dried on 3MM Whatman filter paper.

As positive controls for subsequent hybridisations, 500 pg of each probe was also applied to the membrane. Probes were denatured in 0.4 M NaOH and 10 mM EDTA in a final volume of 100 µL, followed by heating to 100°C for 10 min. An equal volume of ice-cold 2 M ammonium acetate pH 7.0 was added to neutralise the DNA, and the solutions kept on ice until application. DNA was bound to the membrane under gentle vacuum as described for RNA above. Following application 500 µL of 2 x SSC was applied to wash DNA from the well walls. RNA and DNA was fixed to the membrane by oven baking at 80°C for 2 h.

3.2.8.4 Chemiluminescent Detection of mRNA

Chemiluminescent detection of mRNA and rRNA was performed using the ECL Direct Nucleic Acid Labelling and Detection System (ECL kit) (Amersham Pharmacia Biotech

UK Ltd.) according to manufacturer's instructions. For each reaction, 100 ng of DNA probe was labelled with horseradish-peroxidase and hybridised in tubes (Thermo Hybaid, Ashford, Middlesex, UK) overnight to the immobilised RNA in 25 mL hybridisation buffer (Appendix 7.2.6). Following stringency washes, excess 2 x SSC was drained and the membrane placed in a clean plastic tray. Ten mL each of detection reagents 1 and 2 were combined, poured on to the membrane, and incubated for 1 min at room temperature. Excess detection reagent was drained, the membrane placed between clean mylar sheets and pressed to remove air. The mylar-encased membrane sheet was placed in an X-ray film cassette and a sheet of ECL hyperfilm (Amersham Pharmacia Biotech UK Ltd.) placed on top. The cassette was closed and the film exposed for 1 h. Films were developed manually using the Kodak GBX system (Kodak [Australia] Pty., Ltd., Coburg, Vic., Australia).

3.3 Results

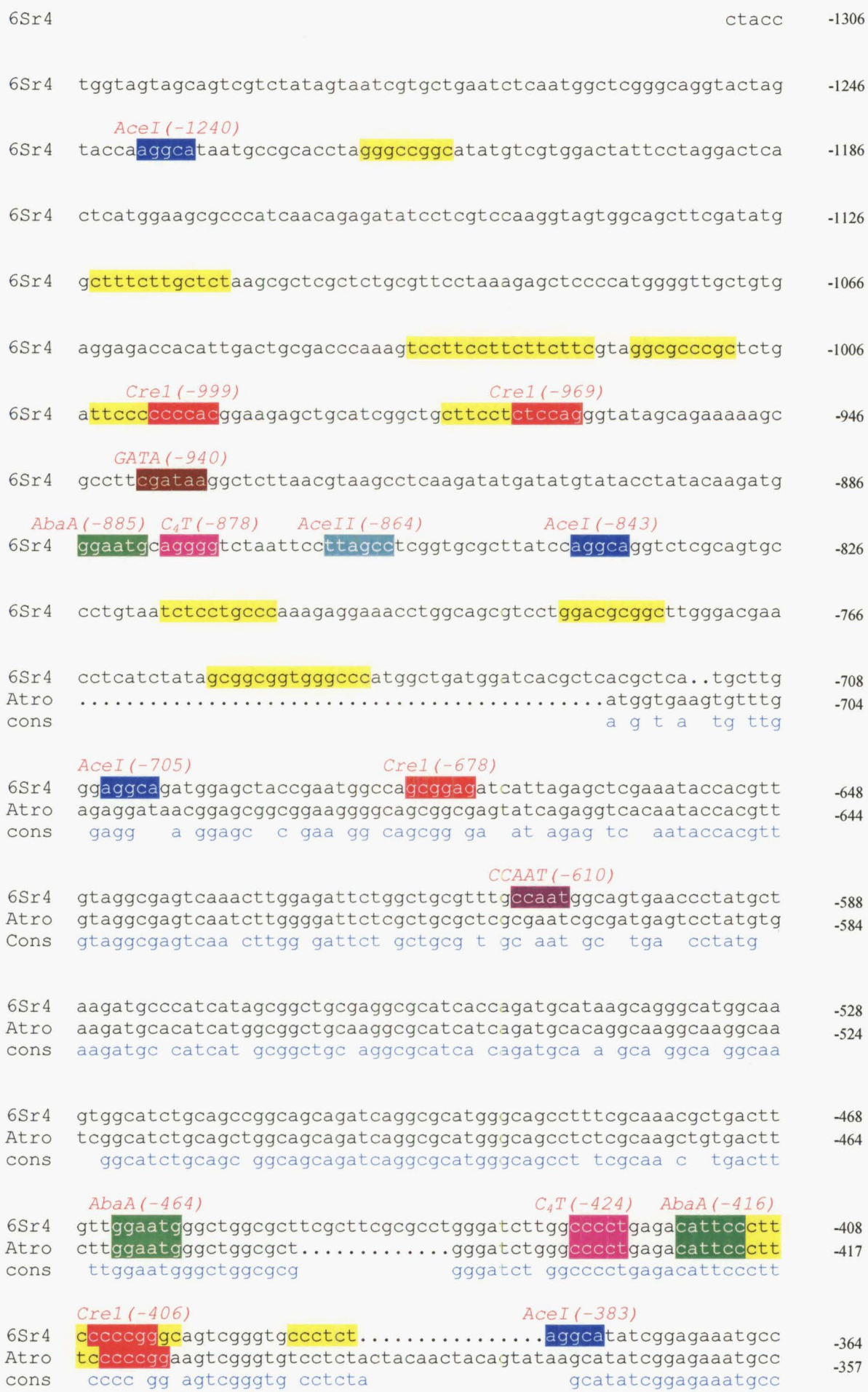
3.3.1 Sequence Analysis of the *chit42* Regulatory Region

3.3.1.1 Isolation of *chit42* from 6Sr4

A 2.69 kb region of the *chit42* gene, which encompassed 1418 bp upstream of the translational start site to 1269 bp of the 1467 bp coding region, was amplified, cloned and sequenced from *T. hamatum* 6Sr4. Initially, primers ECH-315 and ECH-1612 yielded two DNA fragments of approximately 1.7 and 1.3 kb in length. Sequencing revealed the 1.3 kb fragment to have 99.84% nucleotide identity and a 99.41% amino acid identity to *T. hamatum* (Tam61) *chit42* (ascn#: U88560). Using the primer set ECH-1 and ECH-1590, a 1.6 kb region extending from the 5' flanking region to the first 130 nucleotides of the coding region was also cloned and sequenced. This gene fragment had 99.54% identity to *T. hamatum chit42* (U88560).

3.3.1.2 Identification of Putative Regulatory Motifs

The location of previously reported regulatory motifs was established through computer-aided sequence searching (Figure 3.5). A putative transcription start point was identified on the basis of alignment to true start points in *T. atroviride chit42* (P1) (ascn#: Z80358) and *T. virens* (Gv29-8) (AF050098). The motifs appeared to cluster into two domains. Alignment with *T. atroviride* (723 bp) and *T. virens* (255 bp) 5' to the transcription start point (TSP) revealed conserved motifs within the nucleotide region up to 713 bp 5' to the TSP in 6Sr4 *chit42* (Figure 3.5). In addition, a 1998 study (Córtes *et al.*, 1998) detailed regulatory motifs in *T. atroviride* (IMI206040) *chit42* and *prb1* up to approximately 875 and 1040 bp, respectively, 5' to the TSP, however the full nucleotide sequence associated


6Sr4 ctacc -1306
6Sr4 tggtagtagcagtcgtctatagtaatcgtgctgaatctcaatggctcgggcaggtactag -1246
AceI(-1240)
6Sr4 taccaaggcataatgccgcacctagggccggcatatgtcgtggactattcctaggactca -1186
6Sr4 ctcatggaagcgcccatcaacagagatatcctcgtccaaggtagtggcagcttcgatatg -1126
6Sr4 gctttcttgctctaagcgctcgctctgcgttcctaaagagctccccatggggttgctgtg -1066
6Sr4 aggagaccacattgactgcgacccaaagtccttccttcttcttcgtaggcgcccgctctg -1006
Cre1(-999) Cre1(-969)
6Sr4 attcccccccacggaagagctgcatcggctgcttcctctccaggggtatagcagaaaaagc -946
GATA(-940)
6Sr4 gccttcgataaggctcttaacgtaagcctcaagatatgatatgtatacctatacaagatg -886
AbaA(-885) C4T(-878) AceII(-864) AceI(-843)
6Sr4 ggaatgcaggggtctaattccttagcctcggtgcgcttatccaggcaggtctcgcagtgc -826
6Sr4 cctgtaatctcctgcccaaagaggaaacctggcagcgtcctggacgcggcttgggacgaa -766
6Sr4 cctcatctatagcggcggtgggcccatggctgatggatcacgctcacgctca..tgcttg -708
Atro .....atggtgaagtgtttg -704
cons a g t a tg ttg
AceI(-705) Cre1(-678)
6Sr4 ggaggcagatggagctaccgaatggccagcggagatcattagagctcgaaataccacgtt -648
Atro agaggataacggagcggcggaaggggcagcggcgagtatcagaggtcacaataccacgtt -644
cons gagg a ggagc c gaa gg cagcgg ga at agag tc aataccacgtt
CCAAT(-610)
6Sr4 gtaggcgagtcaaacttggagattctggctgcgtttgccaatggcagtgaaccctatgct -588
Atro gtaggcgagtcaatcttggggattctcgctgcgctcgcgaatcgcgatgagtcctatgtg -584
Cons gtaggcgagtcaa cttgg gattct gctgcg t gc aat gc tga cctatg
6Sr4 aagatgcccatcatagcggctgcgaggcgcatcaccagatgcataagcagggcatggcaa -528
Atro aagatgcacatcatggcggctgcaaggcgcatcatcagatgcacaggcaaggcaaggcaa -524
cons aagatgc catcat gcggctgc aggcgcatca cagatgca a gca ggca ggcaa
6Sr4 gtggcatctgcagccggcagcagatcaggcgcatgggcagcctttcgcaaacgctgactt -468
Atro tcggcatctgcagctggcagcagatcaggcgcatgggcagcctctcgcaagctgtgactt -464
cons ggcatctgcagc ggcagcagatcaggcgcatgggcagcct tcgcaa c tgactt
AbaA(-464) C4T(-424) AbaA(-416)
6Sr4 gttggaatgggctggcgcttcgcttcgcgcctgggatcttggccctgagacattccctt -408
Atro cttggaatgggctggcgct.....gggatctgggccctgagacattccctt -417
cons ttggaatgggctggcgcg gggatct ggcccctgagacattccctt
Cre1(-406) AceI(-383)
6Sr4 ccccccggcagtcgggtgccctct.....aggcatatcggagaaatgcc -364
Atro tccccccggaagtcgggtgtcctctactacaactacagtataagcatatcggagaaatgcc -357
cons cccc gg agtcgggtg cctcta gcatatcggagaaatgcc

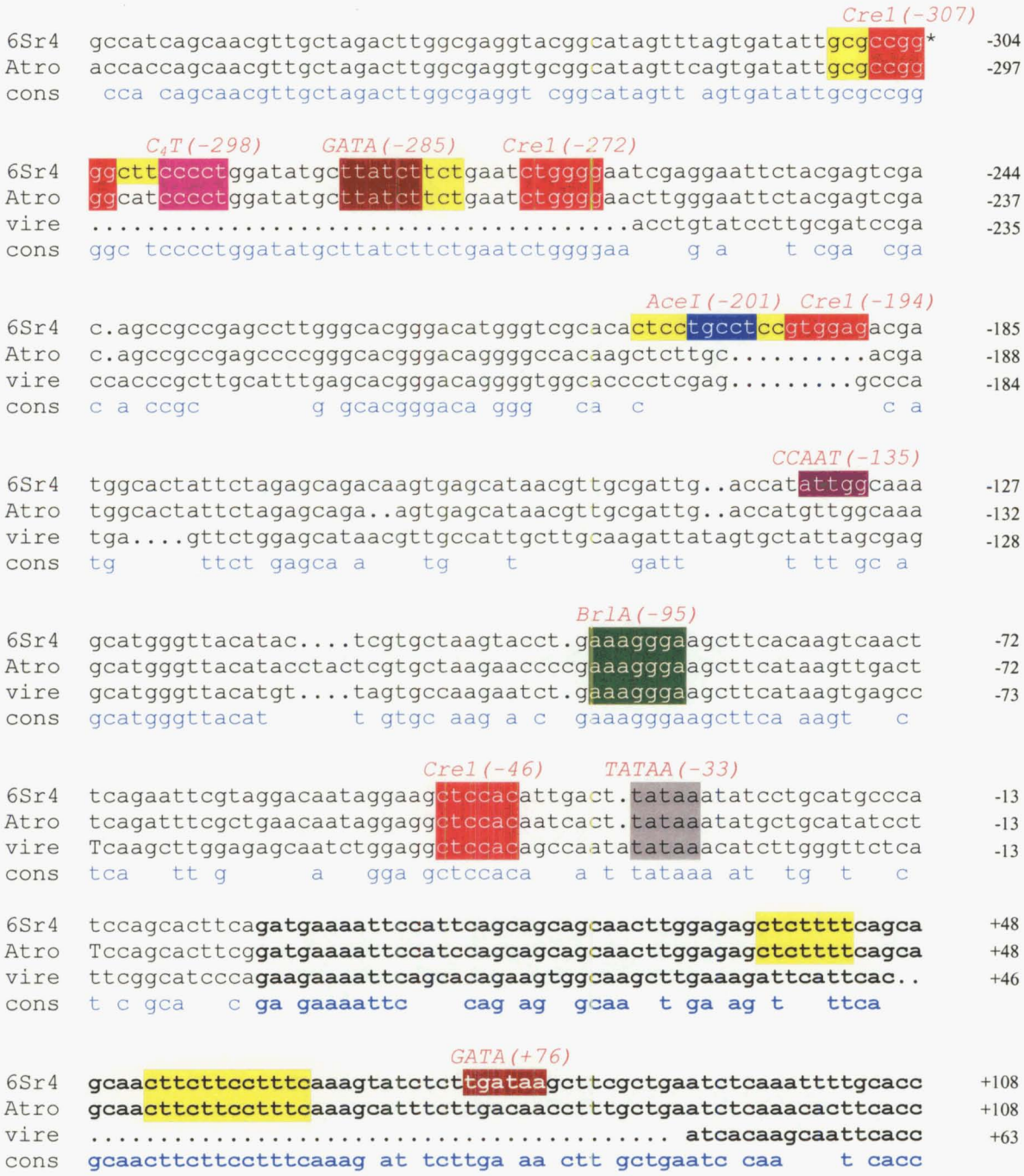


Figure 3.5. Alignment of *chit42* upstream of the ATG start codon. Atro = *T. atroviride* (ascn#: Z80358); Viro = *T. virens* (ascn#: AF050098). Numbering is relative to the tentative TSP +1, based on alignment. The 5' untranslated region is in bold. GC- and CT-rich elements are shaded in yellow. Where two motifs overlap, bases are in upper case.
* motif continues on next line

with this study has not been published on GenBank and was therefore unavailable for sequence alignment.

Common Eukaryotic Elements

A probable transcription site at position +1 was identified based on alignment to *T. atroviride* strain IMI206040 (ascn#: X79381) and *T. virens* (ascn#: AF050098). A conserved TATAA box motif was identified at position –33. No CCAAT boxes were found proximal to the transcription start point, however, motifs were found at –135 and –610. Neither of these were conserved with the *chit42* sequences available for alignment. Three consensus GATA motifs (5'-HGATAR-3') were identified in *chit42* (+77, -284, -939). Position –284 was conserved in *T. atroviride* (Z80358). Multiple CT- and GC-rich regions ranging from 6-14 bp in length were found singly and in association with other putative regulatory motifs.

Regulatory Motifs Identified in Filamentous Fungi

Eight putative *Cre1* binding motifs (5'-SYGGRG-3') were identified in 6Sr4 *chit42*. Only the *Cre1* site at position -46 was conserved with both *T. atroviride* (Z80358 and X79381) and *T. virens* (AF050098). *Cre1* motifs at -272, -307 and -406 were conserved with *T. atroviride* (Z80358). One putative *BrlA* binding box (5'-MRAGGGR-3') was present at –95 and this was conserved with *T. atroviride* and *T. virens*. Three putative *AbaA* binding motifs (5'-CATTCY-3') were present in the regulatory region of *chit42*. *AbaA* at positions –416 and –464 were conserved with *T. atroviride* (Z80358). Position –416 overlapped the 5' end of a 12 bp CT-element which contained the –406 *Cre1* motif. Three conserved C_4T motifs were identified at –298, -424 and –878. Positions –424 and –878 lay 5' to *AceI* motifs. Five putative *AceI* (5'-AGGCA-3') and one *AceII* (5'-GGCTAA-3) motifs were present in the 5' region of *chit42*. *AceI* at –843 and *AceII* at –864 lay only 21 bases apart. None of these motifs were conserved with *T. atroviride* (Z80358).

3.3.2 Sequence Analysis of the *prb1* Regulatory Region

3.3.2.1 Isolation of *prb1* from 6Sr4

Primers previously designed to amplify *prb1* from *T. atroviride* were successfully used to isolate the orthologous gene from *T. hamatum* isolate 6Sr4. Using the primers PRB-F35 and PRB-R1212 a single band of approximately 1.2 kb was amplified and sequenced revealing an 88.75% nucleotide identity and a 96.4% amino acid identity to *T. atroviride* strain IMI206040 *prb1* (M87518). Inverse primers, I-PRB-509 and I-PRB-1067 amplified

a 2 kb inverse PCR product from a *Hind*III digestion of 6Sr4. Digestion of the clonal PCR product with *Hind*III revealed two bands of approximately 800 bp and 1.2 kb. Subsequent sequencing showed the 800 bp band was the 5' flanking region and the 1.2 kb band was the 3' flanking region. The complete 2527 bp sequence was composed of 735 bp 5' to the translational start site, the complete 1358 bp coding region and 433 bp 3' to the stop codon.

3.3.2.2 Identification of Putative Regulatory Sites

Important regulatory sites and motifs were established for *prb1* as described for *chit42* above (Figure 3.6). The 705 bp *T.* hamatum 6Sr4 regulatory region 5' to the TSP was aligned with 394 bp of *T. atroviride* strain IMI206040 (ascn#: M87518) (Figure 3.6). A description of regulatory motifs up to 1050 bp 5' to the TSP in *T. atroviride* was also available (Córtes *et al.*, 1998).

Common Eukaryotic Elements

The transcription initiation site (position +1) was predicted by alignment with *T. atroviride* (ascn#: M87518). The first 8 bp of the 5' UTR, including a 7 bp CT-rich element, were 100% identical.

The promoter region of *prb1* lacked a consensus 5'-TATAA-3' motif 5' proximal to the TSP, however a reverse TATAA-like motif within a 9 bp AT-rich region was present at –42 and was conserved with *T. atroviride*. A consensus TATAA box motif was present within the 5' untranslated region, within a 10 bp AT-rich region (position +12) and was not conserved with *T. atroviride*. As with chit42 no CCAAT box motifs were present proximal to the TSP, however two were present at –575 and –601. Four consensus GATA sites were identified in 6Sr4 *prb1* at –108, -178, -310 and –563, however only –108 was conserved with *T. atroviride*. Eight CT- and GC-rich regions ranging from 7 to 17 bp in length were identified in *prb1*. As described earlier a 7 bp CT-element was located at +1 and was conserved with *T. atroviride*.

Regulatory Motifs Identified in Filamentous Fungi

Unlike *chit42* only one *Cre1* motif was present in the *prb1* 5' region (-315) and this was not conserved with *T. atroviride* (ascn#: M87518). No *BrlA*, *AbaA* or C_4T motifs were identified in the regulatory region, however one putative *AceI* and two *AceII* sites were identified.

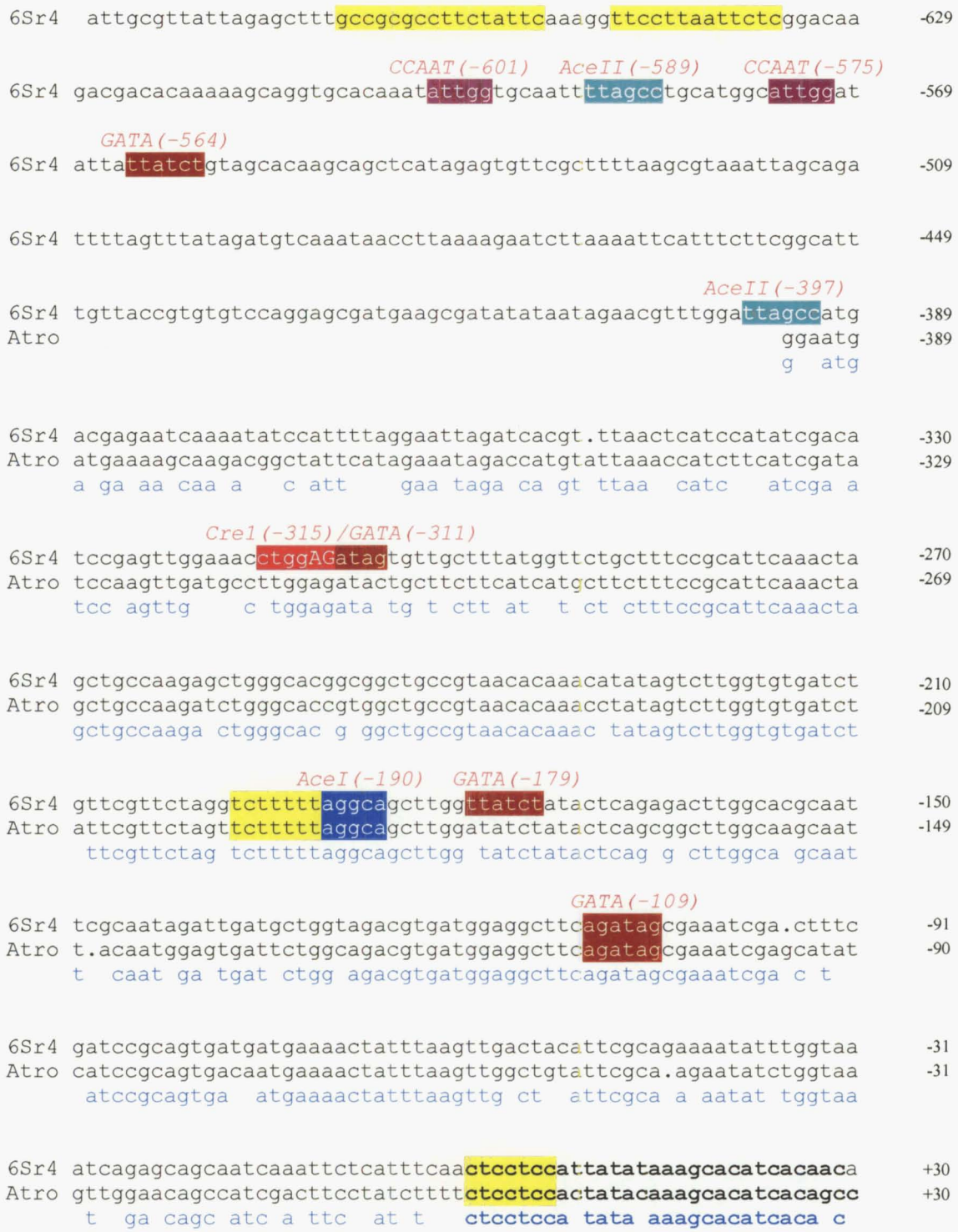


Figure 3.6. Alignment of *prb1* upstream of the ATG start codon. Atro = *T. atroviride* (ascn#: M87518). Numbering is relative to the tentative TSP +1, based on alignment. The 5' untranslated region is in bold. GC- and CT-rich elements are shaded in yellow. Where two motifs overlap, bases are in upper case.
* motif continues on next line

3.3.3 Sequence Analysis of the *xbg1.3-110* Regulatory Region

3.3.3.1 Isolation of *xbg1.3-110* from 6Sr4

Using a combination of degenerate, inverse and specific PCR, *xbg1.3-110*, a *lam1.3* orthologue, was cloned and sequenced from *T. hamatum* 6Sr4. Amplification with degenerate primers XBGp394 and XBG535p generated multiple amplimers, from which a product of the predicted (485 bp) size was cloned and sequenced. Blastn searching showed a 86.67% nucleotide identity and a 90.7% amino acid identity to *T. harzianum* strain T-Y *lam1.3* (ascn#: AJ002397). Multiple clones were used to extend the 485 bp sequence. Combined sequence data comprised a 3968 bp region of *xbg1.3-110* which extended from 1033 bp 5' to the start codon to 2935 bp of the predicted 3743 bp coding region.

3.3.3.2 Identification of Putative Regulatory Motifs

Regulatory sites and motifs were identified in *xbg1.3-110* as described for *chit42* and *prb1* above (Figure 3.7). Like chit42, the motifs appeared to cluster into two domains. Sequence information for alignments was only available from *T. harzianum* T-Y (ascn#: AJ002397) (Figure 3.7) and extended 58 bp 5' to the TSP.

Common Eukaryotic Elements

A probable transcription start point was selected on the basis of alignment with the multiple initiation sites reported for *T. harzianum* (Cohen-Kupicek *et al.*, 1999). A typical TATAA-box motif was identified at –54, and this was largely conserved with *T. harzianum*. No CCAAT-boxes were present proximal to the transcription start point, however a CCAAT motif was present at position –588. Five putative GATA sites were identified. Conservation with *T. harzianum* was only observed for the motifs at +63 and +54. Multiple CT-rich and GC-rich regions ranging from 6-21 bp in length were found on their own or in association with regulatory motifs.

Regulatory Motifs Identified in Filamentous Fungi

Eight putative *Cre1* motifs were identified in the regulatory region of *T. hamatum* 6Sr4 *xbg1.3-110*. Position –216 overlapped a *BrlA* box at –217 and position –67 overlapped a GATA site. Two *BrlA* motifs were identified in the regulatory region and these lay near to the two *AbaA* motifs. Two *AceI* and one *AceII* binding sites were identified in the 5' region.

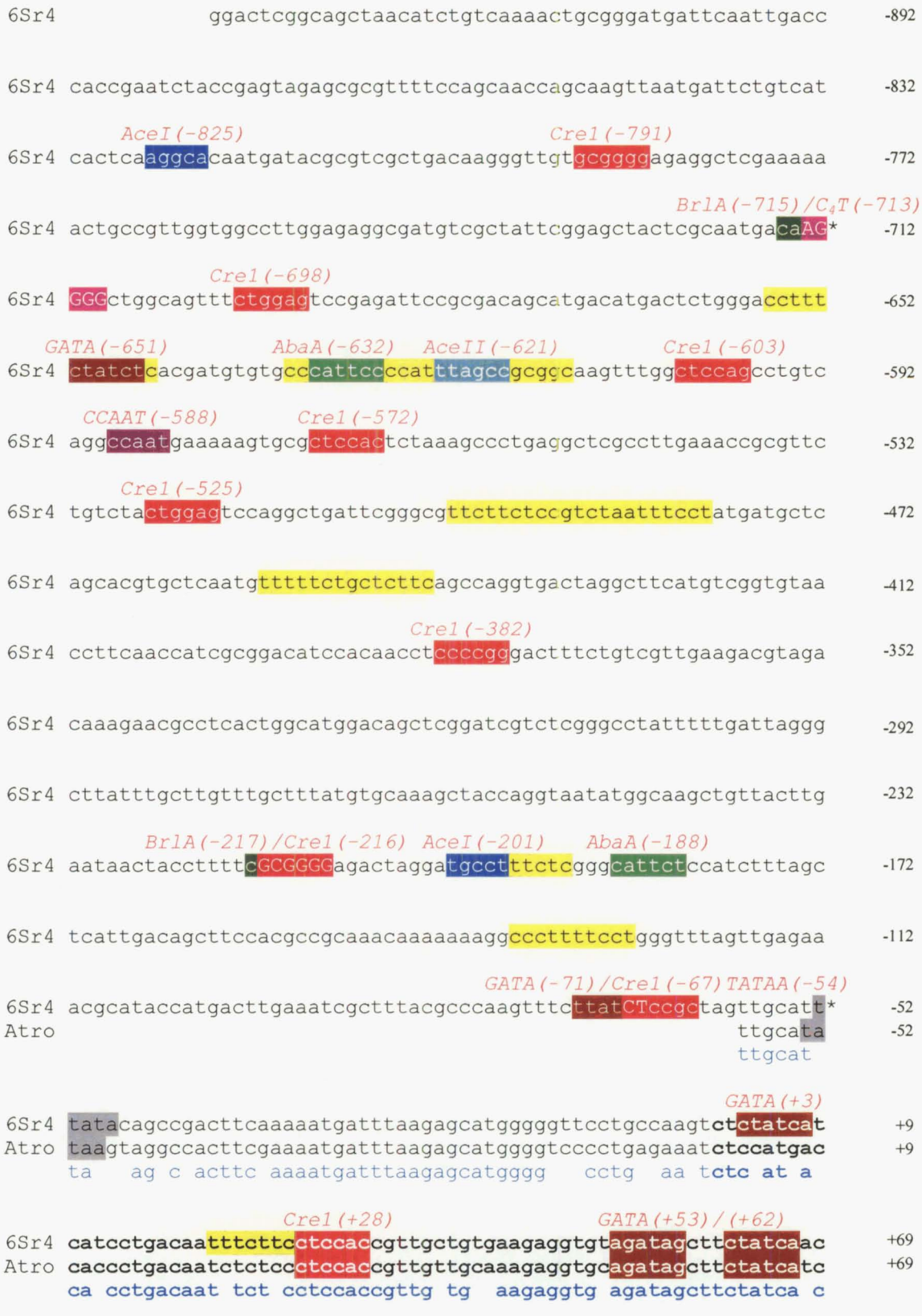


Figure 3.7. Alignment of *xbg1.3-110* upstream of the ATG start codon. Harz = *T. harzianum* (ascn#: AJ002397). Numbering is relative to the tentative TSP +1, based on alignment. The 5' untranslated region is in bold. GC- and CT-rich elements are shaded in yellow. Where two motifs overlap, bases are in upper case.
* motif continues on next line

3.3.4 Identity Between the Regulatory Regions of *chit42*, *prb1* and *xbg1.3-110*

From the preceding sections it was apparent that similarity existed in the organisation of the regulatory regions of *chit42*, *prb1* and *xbg1.3-110*. As *chit42*, *prb1* and *xbg1.3-110* have all been implicated in mycoparasitism, and are inducible on a wide variety of carbon sources, alignments of the promoter regions was performed to identify putative binding sites for undescribed transcription factors. Multiple regions of 100% identity 6 bp or greater in length were present (Appendix 7.6), and many of these were conserved in both sequence and position (Figure 3.8).

The regulatory regions of *chit42* and *prb1* shared several identities. In particular, a cluster of identities between *chit42* and *prb1* (CP-5, CP-6, CP-7, CP-8) lay in exactly the same spatial arrangement (Figure 3.8). All four identities were conserved with *T. atroviride*, whereas only CP-8 was 100% conserved in *T. virens*. Three of the four identities had been described previously in *T. atroviride* (MYC3-MYC1) where a role in mycoparasitic gene induction was postulated (Córtes *et al.*, 1998).

CP-5 (MYC3) was conserved with *T. atroviride* strain IMI206040 *chit42* (ascn#: X79381), but not fully with strain P1 (Z80358), where a C to T substitution occurred in the first base (Figure 3.9). The region immediately 5' to CP-6 was also highly conserved between genes and species (90%). CP-2 and CP-3 lay next to or overlapped each other in *chit42* and *prb1* and aligned to MYC4 in *T. atroviride* (Córtes *et al.*, 1998).

Figure 3.9. Alignment of CP-5 in *T. atroviride* and *T. hamatum chit42* and *prb1* 5' region. 100% identity across all five sequences is in blue. Identity across four sequences is in red; across three or two sequences is in green. CP-5 is indicated in bold.

Four of the six *Cre1* motifs presented for *xbg1.3-110* had equidistant counterparts in *chit42*, and the single site in *prb1* aligned positionally to *chit42*. Two motifs were conserved in position between all genes: the CCAAT box motif at approximately –580,

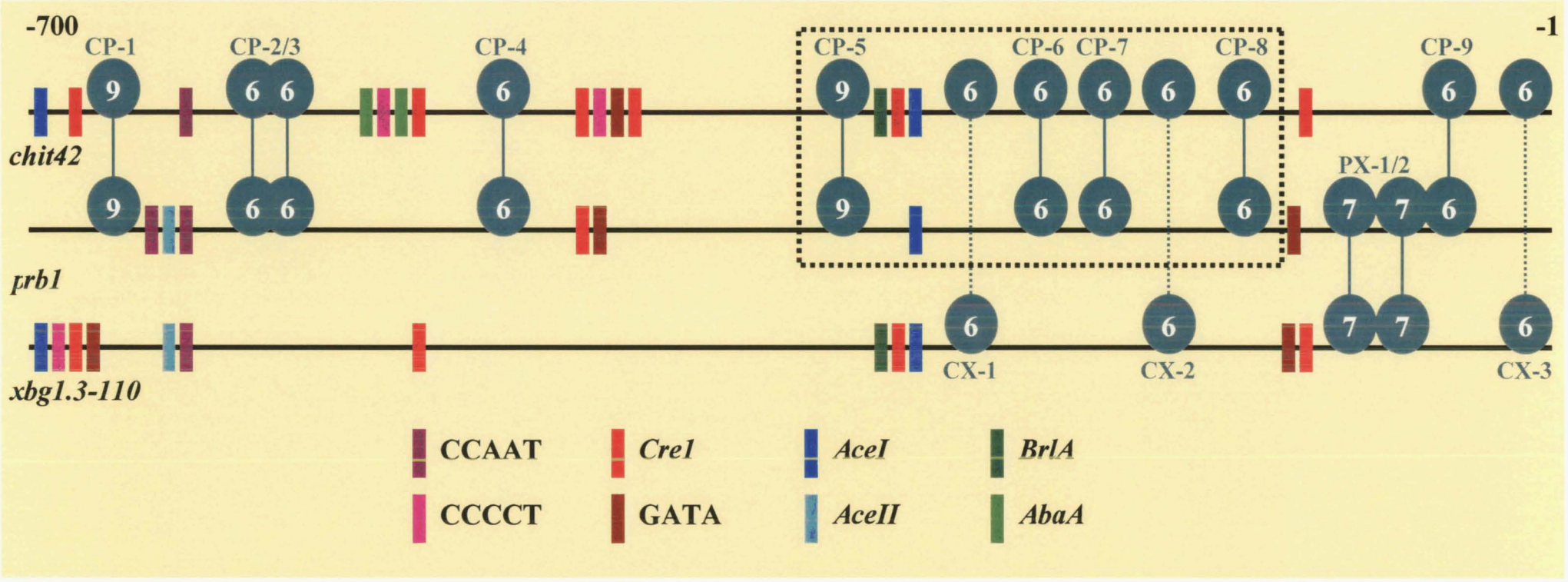


Figure 3.8. Alignment of the first 700 bp 5' to the TSP in *T. hamatum chit42*, *prb1* and *xbg1.3-110*. Motifs that are line with each are equidistant to the TSP within 50 bp. Circles represent 100% idenitities between promoters. The numbers inside the circles represent the length of identity. The cluster of CP-5 to CP-8 is boxed.

and the *AceI* motif at approximately –200. The equidistant *AceII* motifs at –589 and –621 in *prb1* and *xbg1.3-110* lay within 50 bp to the conserved CCAAT box motif.

3.3.5 Gene Expression analysis of *chit42*, *prb1* an *xbg1.3-110*

Strong sequence identity between the regulatory regions of *chit42* and *prb1* led to the prediction that they shared common regulatory pathways. To test this hypothesis, gene expression patterns of *chit42*, *prb1* and *xbg1.3-110* were analysed in response to high glycerol and in confrontation with *Sclerotinia sclerotiorum.*

3.3.5.1 Gene Expression in Response to High Glycerol

No mRNA was obtained from preparations of the treatments containing chitin. The chitin used in the pilot trial was purified from practical grade (SIGMA: C-7170) using the HCl extraction method (Skujins *et al.*, 1965), and the possibility existed that errors in purification may have affected RNA extractions.

In contrast to reported activity in *T. atroviride*, *T. hamatum chit42* and *prb1* were strongly induced in the minimal media (MM) + 2% glycerol control of the pilot experiment (results not shown); no induction of *xbg1.3-110* was detected. Glycerol was included in the medium to alleviate induction by starvation. Neither culture was able to grow in the absence of glycerol, whereas in 2% glycerol both cultures increased in size throughout the experiment, indicating metabolism of the available carbon. In *T. atroviride* no induction of *chit42* and only low-level induction of *prb1* were reported (Córtes *et al.*, 1998). The strong induction in the presence of glycerol was investigated further and these results are shown in Figure 3.10.

Further investigation showed that expression of both *T. hamatum chit42* and *prb1* peaked at 48 h and had noticeably declined by 96 h. *Xbg1.3-110* expression was not detected at any time interval, even at basal levels. In contrast, when 6Sr4 was grown in MM + 2% glycerol supplemented with 0.5% laminarin, *xbg1.3-110* was strongly induced at 48 hours (Figure 3.10). *T. harzianum chit42* or *prb1* expression was not detected at any time interval indicating *T. hamatum chit42* and *prb1* are regulated differently to their *T. harzianum* orthologues in response to high glycerol.

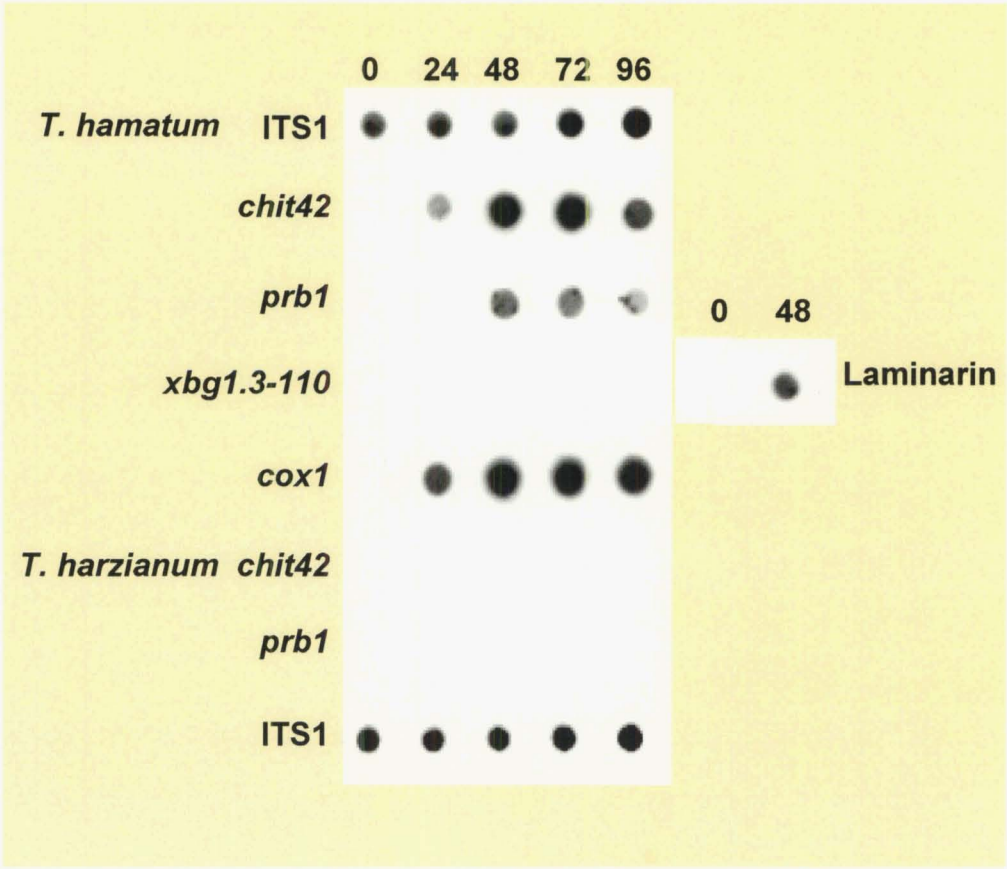


Figure 3.10. Northern dot-blot analysis of *chit42*, *prb1 and xbg1.3-110* gene expression in response to glycerol, and *xbg1.3-110* in response to glycerol and laminarin at 48 h.

3.3.5.2 Direct Confrontation Assay

Orthologues of the three genes characterised in this study have been induced during confrontation with fungal plant pathogens. To investigate whether this was also true for *T. hamatum* 6Sr4, gene expression patterns during confrontation with *S. sclerotiorum* were analysed (Figure 3.11).

Results showed that *Trichoderma hamatum chit42* and *prb1* were moderately induced during confrontation with *S. sclerotiorum*, however no induction of *xbg1.3-110* was detected at any stage. Induction was strongest in the *T. hamatum* vs. *S. sclerotiorum* overlap samples; slight induction was observed during confrontation of 6Sr4 with itself.

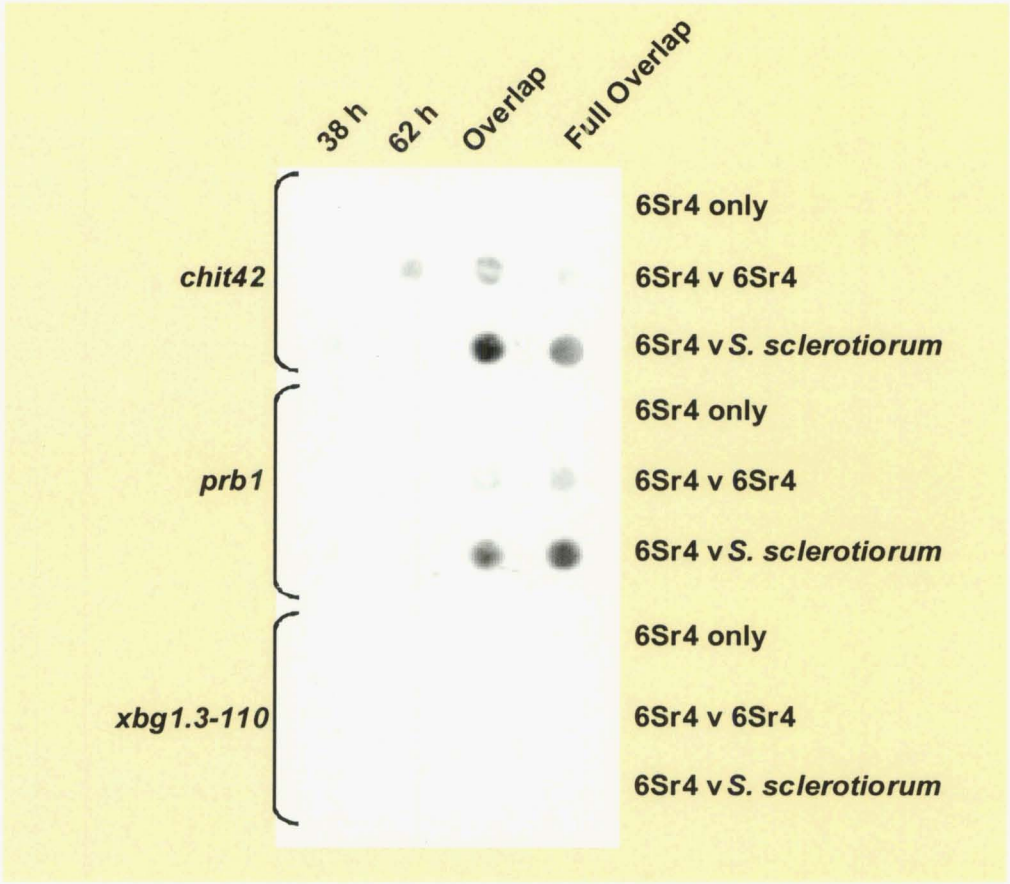


Figure 3.11 Northern dot-blot analysis of *chit42*, *prb1* and *xbg1.3-110* gene expression during mycoparasitism.

3.3.6 Results Summary

Of the three genes characterised, *chit42* and *prb1* shared the most sequence identity in the promoter/enhancer region as well as in gene expression patterns under two inducing conditions. The expression patterns of all genes were different to those reported previously for gene orthologues from other species. Contrasting gene expression patterns were demonstrated between *T. hamatum* and *T. harzianum* in response to glycerol as the sole carbon source.

On the basis of differential expression patterns, two subsets of putative binding motifs for induction in response to glycerol and during mycoparasitism were derived from the sequence identities presented in Figure 8. CP-1 was not conserved with *T. atroviride chit42*

and *prb1*, nor with *T. hamatum xbg1.3-110.* As none of these genes are strongly induced in response to high glycerol, this suggested a possible role in glycerol response. CP-2 and CP-3 (MYC4) and CP-5 (MYC3), CP-6, CP-7 (MYC2) and CP-8 (MYC1) are all conserved with *T. atroviride* but not with *T. hamatum xbg1.3-110* which suggested a possible role in mycoparasitism.

3.4 Discussion

3.4.1 Structural Analysis of the *chit42*, *prb1* and *xbg1.3-110* Regulatory Regions

Eukaryotic genes are regulated by two groups of cis-acting elements termed promoters and enhancers. Distinction between these two groups has been on the basis of binding and function. Promoter elements act together to direct efficient and accurate initiation of transcription by binding of the RNA polymerase complex (holoenzyme) and are considered essential, whereas enhancer elements increase (or repress) the rate of transcription in response to stimuli (Maniatis *et al.*, 1987). The regulatory motifs in the upstream regions of *chit42* and *xbg1.3-110* from *T. hamatum* 6Sr4 clustered in two domains, which may represent promoter and enhancer regions. Insufficient sequence data was available to determine whether a domain-like structure occurred in *prb1.*

The TATAA box motif has been established as a key promoter structure. Transcription has been shown to begin with the binding of the TATA-binding protein (TBP) to the TATAA box (White and Jackson, 1992). Both *T. hamatum chit42* and *xbg1.3-110* have typical promoter structures. Consensus TATAA motifs were found at –33 and –54 in *chit42* and *xbg1.3-110*, respectively, and both were conserved in other *Trichoderma.* The absence of clearly recognisable TATA-box and the presence of a highly conserved CT-element at the site of transcription initiation suggested that *prb1* may have a TATA-less promoter. Transcription initiation in the absence of a TATA-box has been demonstrated to be coordinated through the Inr, a sequence element encompassing the start point. The area around +1 in *prb1* was similar in sequence to Inr elements of TATA-less promoters, that is CT-rich regions flanking a CA motif at +1 (Weis and Reinberg, 1992; Chen and Roxby, 1997).

CCAAT box motifs act as enhancers in all eukaryotes. A variety of CCAAT binding factors have been identified in higher cells, however only the HAP complex has been demonstrated to bind CCAAT in fungi (Brakhage *et al.*, 1999). In *T. reesei*, HAP binding to a CCAAT box is essential for coordinated binding of a specific regulator to the *cbh2*

(cellobiohydrolase) promoter, which disrupts downstream nucleosome positioning making the TATA-box accessible (Susanne Zeilinger, pers. comm.). In addition, the HAP complex also coordinates binding to *AceII* in *xyn2*, a xylanase gene implicated in mycoparasitism (Robert Mach, pers. comm.). The relevance of the CCAAT-box motif in *T. hamatum* is unknown, however the conservation at about -600 between genes, and the proximity of *AceII* motifs in *prb1* and *xbg1.3-110* may indicate a role for the HAP complex in a common pathway.

Functional CCAAT boxes have traditionally been found within the first 200 bp 5' to the transcription start point. Of the three genes characterised in this study, only *chit42* had a CCAAT box within this region (-135). The lack of conservation with *T. atroviride* or *T. virens*, suggested this motif did not play a key promoter role in *chit42*. This contrasts with previous gene characterisation work by Cortés *et al.* (1998), which reported CCAAT boxes at approximately –60 in both *chit42* and *prb1* in *T. atroviride* strain IMI206040. Closer inspection of the sequences revealed the motifs they had identified were CAAT not the consensus CCAAT.

GATA binding factors are involved in regulation of major physiological processes, such as global nitrogen repression and derepression, where their major effect is alteration of nucleosome structure (Scazzocchio, 2000). More recently, *T. atroviride prb1* has been demonstrated to be under the control of nitrogen catabolite repression (NCR), coordinated by binding to the GATA site at -108 (Carlos Cortés, pers. comm.). As this motif is conserved in *T. hamatum prb1*, it is likely this gene is also under the control of NCR.

Carbon catabolite repression is a common regulatory mechanism of both prokaryotes and eukaryotes, and in *Trichoderma* it negatively regulates a variety of genes implicated in mycoparasitism. Negative regulation of *chit42*, *prb1* and *xbg1.3-110* by carbon catabolite repression has been well documented, implicating a role for these genes in metabolism of alternate carbon sources, of which mycoparasitism is a part. *Cre1* motifs were identified in the regulatory regions of *T. hamatum chit42*, *prb1* and *xbg1.3-110*.

Several *Cre1* motifs in 6Sr4 *prb1* and *xbg1.3-110* were conserved in position with motifs identified in *chit42*. In addition GATA motifs were found near to or overlapping the *Cre1* motif in *prb1* and two of the four motifs conserved in position between *chit42* and *xbg1.3-110*. During mycoparasitism Cre1 is dephosphylated which prevents binding (R.L. Mach &

C.P. Kubicek (unpublished) in Kubicek-Pranz, 1998) and an unidentified protein/s binds to the promoter in the vicinity of the *Cre1* motif. This unidentified protein may be a GATA transcription factor, or an associated protein.

BrlA and AbaA are positive regulators of conidiation in *Aspergillus* (Chang and Timberlake, 1992; Andrianopolus and Timberlake, 1994). Binding motifs for both of these proteins were identified in *T. hamatum chit42* and *xbg1.3-110*, which implicated a role for these genes in light-induced sporulation. During sporulation, polysaccharide degrading enzymes such as Chit42 and Xbg1.3-110 are involved in cellular differentiation. Neither motif was present in *prb1*, suggesting this gene is not induced during sporulation.

In *S. cerevisiae* stress response is mediated by binding of Msn2p/Msn4p to a C_4T motif. Orthologues of *msn2/msn4* have been isolated from *T. atroviride* and demonstrated to bind C_4T sequences. In addition, studies have demonstrated *T. atroviride* P1 *chit42* to be stress-inducible and C_4T motifs identified within the *chit42* regulatory region. Alignment with *T. atroviride* P1 *chit42* revealed conservation with two of three C_4T motifs identified in 6Sr4. Only the third upstream motif was reported in *T. atroviride* IMI206040 (Cortés *et al.*, 1998). Therefore, in *T. hamatum*, *chit42* is likely to be stress-induced.

Binding of AceI and AceII to the *cbh1* promoter and AceII to the *xyn2* promoter of *T. reesei* is essential for full gene expression in response to their relative substrates, however the role of these motifs in other genes (if active) is unknown. *AceI* and *AceII* motifs were identified in the regulatory regions of *T. hamatum chit42*, *prb1* and *xbg1.3-110*. Only the single *AceI* motif in *prb1* was conserved with other *Trichoderma* species. Interestingly, the *AceI* motif in *prb1* was conserved in position with *AceI* motifs in *chit42* and *xbg1.3-110*. As mycoparasitism of *Aphanomyces euteiches*, an Oomycete, has been observed by *T. hamatum* 6Sr4 (Chapter 1.0), it is possible that induction by the cellulose-containing *A. euteiches* may have involved *AceI* binding.

3.4.2 Comparison of the Genetic Regulation of *chit42*, *prb1* and *xbg1.3-110*

A common approach to sequence analysis has been comparison of gene orthologues in related species or co-regulated genes in the same species. In this study, both approaches were taken. In recent studies, Cortés *et al.* (1998) compared the 5' flanking regions of *T. atroviride chit42* and *prb1*, and identified four putative binding sites (MYC4 – MYC1) for a global inducer of mycoparasitism. Mutational knockout of MYC4 resulted in a decrease

in the rate of transcription, which implied that this factor binds to an enhancer type element (Córtes *et al.*, 1998). These four MYC motifs were conserved in the *T. hamatum* orthologues, which substantiates the hypothesis that they represent binding sites for regulatory proteins. In this study CP5 (MYC3), CP6, CP7 (MYC2) and CP8 (MYC1) formed a highly conserved cluster proximal to the transcription start site that spanned exactly 142 bp in each gene. The 6 bp identity CP6 was also conserved in *T. atroviride chit42* and *prb1*, however it had not been identified in Cortés *et al.* (1998). This study suggests that an additional motif MYC5 (CP6) should be added to the evolving model for the mycoparasitism specific binding region (Figure 3.12) proposed by Lorito *et al.* (1996).

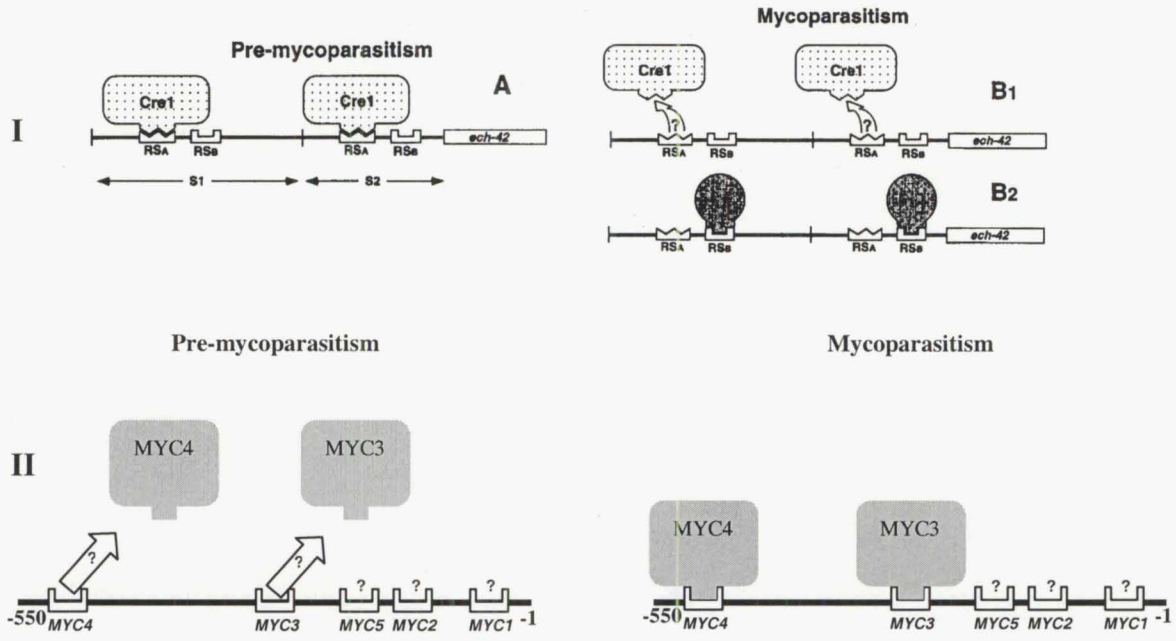


Figure 3.12. Evolving model for the mycoparasitism specific binding region of *Trichoderma chit42* and *prb1*. **I:** Model from Lorito *et al.* (1996). **II:** This study.

Each motif within the cluster lay exactly the same distance apart in both *chit42* and *prb1*. As upstream elements associated with TBP and holoenzyme binding have been shown to act in a position-dependent manner (Maniatis *et al.*, 1987), this implies possible involvement with a transcriptional complex. Small insertions and deletions have been shown to disrupt binding and prevent transcription, therefore, the strong positional conservation of these four elements between *chit42* and *prb1* may be due to functional constraints.

Degeneracy within binding sites for regulatory proteins is a common feature, for example the Cre1 binding motif: 5'-SYGGRG-3'. Research has shown that regulatory proteins recognise specific conformations within the DNA sequence, rather than individual nucleotide residues (Nussinov, 1990), therefore, nucleotide substitutions that have no effect on structure do not affect binding. Of the four motifs within the conserved cluster, only CP8 (MYC1) was 100% conserved in *T. virens*. Loss of conservation in the other three motifs was due to purine substitutions (A/G). As purines exhibit the same structure and charge, substitutions may not confer changes in DNA shape, therefore, it is possible these substitutions do not affect binding.

In the model proposed by Cortés *et al* (1998) for the regulation of mycoparasitism it was suggested an unidentified protein bound to MYC3 in the mycoparasitic state, and Cre1 bound to an overlapping *Cre1* motif in the non-mycoparasitic state. Whilst MYC3 (CP-5) was conserved in *T. hamatum*, the overlapping *Cre1* motif was not. This suggests that the *Cre1* motif adjacent to MYC3 in *T. atroviride* is not involved in regulation of mycoparasitism.

The role of MYC1-MYC5 in mycoparasitism is supported by the expression of *chit42* and *prb1* in the substrate and confrontation assays. Current research has demonstrated that genes expressed during mycoparasitism are induced in response to a variety of carbon sources and in confrontation with various fungal pathogens (Mach *et al.*, 1999; Cortés *et al.*, 1998; Geremia *et al.*, 1993). In this study, expression of *T. hamatum* 6Sr4 *chit42* and *prb1* was up-regulated in response to high glycerol and in confrontation with *S. sclerotiorum.*

The response of *T. hamatum chit42* and *prb1* to high glycerol was different to that reported for *T. atroviride* (Cortés *et al.*, 1998) and observed for *T. harzianum* (this study) and is therefore an example of interspecies differential regulation. However, other non-glucose carbon sources, such as V8 juice directly enhance chitinase protein activity in both *T. atroviride* (P1) and *T. virens* (Tronsmo and Harman, 1992). In the presence of both chitin and V8 juice chitinase production followed similar patterns in both isolates, however *T. virens* produced substantially lower yields to *T. atroviride*, also suggesting differential regulation.

Without a good control, it was difficult to conclusively determine that glycerol was the inducer in the submerged culture assays. It is possible asparagine may have induced the genes, however the same level was present in the confrontation assay and the absence of *chit42* and *prb1* gene expression in the 6Sr4 alone control suggests asparagine was not the inducer. As the genes have also been implicated in cellular differentiation and growth, it is possible that induction of 6Sr4 *chit42* and *prb1* were in response to growth-stage regulation of expression. However, the very closely related *T. harzianum* showed no induction at any time despite undergoing parallel growth and differentiation. For this reason it is unlikely that growth stage specific regulation is the cause of the increased *chit42* and *prb1* expression.

Although strong mycoparasitism has been observed by 6Sr4 towards *S. sclerotiorum* (Chapter 1), only moderate induction of *chit42* and *prb1* was observed during confrontation. Similar results have occurred in *T. atroviride* (IMI206040) confronting *Rhizoctonia solani* using the same direct confrontation assay (Carsolio *et al*, 1994; Flores *et al*, 1997). In contrast, both *T. atroviride chit42* and *prb1* were strongly induced in the same assay prior to overlap when physical contact was prevented (Cortés *et* al, 1998; Kullnig *et* al, 2000). In the non-contact assay the host pathogen was inoculated 36 h prior to the *Trichoderma* (Alfredo Herrera-Estrella, pers. comm.). Therefore, whilst the latter studies demonstrated that expression could occur without physical contact, strong induction prior to overlap was likely a result of host pre-inoculation.

Further support for the involvement of MYC1-MYC5 in mycoparasitism was provided by the *xbg1.3-110* expression studies. In contrast to *chit42* and *prb1*, *T. hamatum xbg1.3-110* was not up-regulated in the substrate or confrontation assays. This was surprising, as glycerol has been demonstrated to be a substrate for β-glucanases (Sivan and Chet, 1989) and all three genes have been previously implicated in mycoparasitism (Cortés *et al.*, 1998; Cohen-Kupiec *et al.*, 1999). Gene expression of many ß-1,3- and 1,6-glucanases on fungal cell walls is well documented (Chapter 1.0), however gene expression during confrontation has not been reported to date. The lack of *xbg1.3-110* expression during confrontation and in response to glycerol, demonstrated that it did not share the same induction pathways as *chit42* and *prb1* and did not play a role in initial mycoparasitism of *S. sclerotiorum.* This data supports the results of the sequence analysis, where greater similarity was observed between *chit42* and *prb1*, and MYC1-MYC5 were absent in *xbg1.3-110.*

It is possible that the absence of *xbg1.3-110* expression during confrontation may have been host specific. Indeed, differential expression of ß-1,3-glucanases implicated in mycoparasitism has been observed in relation to different pathogens. Vázquez-Garcidueñas *et al* (1998) demonstrated that *T. atroviride* ß-1,3-glucanase production correlated with the amount of ß-1,3-glucan present in the fungal cell walls incorporated into the medium. Cell walls from *Mucor rouxii*, which have no detectable ß-1,3-glucan, elicited the lowest response, whereas *S. cerevisiae*, with 55% ß-1,3-glucan, elicited the highest response. Cohen-Kupiec *et al* (1999) reported a 110 kDa protein present in protein samples extracted from confrontation of *T. harzianum* with *R. solani* and *Sclerotium rolfsii*, and concluded that it was *xbg1.3-110*. Both *R. solani* and *S. rolfsii* are basidiomycetes, whereas *S. sclerotiorum*, an ascomycete, was used in this study. It is possible that the response of *xbg1.3-110* during confrontation was due to the choice of pathogen.

3.4.3 Implications For Biological Control

The data presented in this study supports current research which suggests the existence of global inducers of mycoparasitism. In both *T. atroviride* (Cortés *et al.*, 1998) and *T. hamatum* (this study), *chit42* and *prb1* are up-regulated during mycoparasitism, and in both cases response is probably due to the elements MYC1-MYC5. The absence of MYC1-MYC5 in *T. hamatum xbg1.3-110* and absence of gene expression during confrontation further supports this hypothesis.

Strong induction in response to glycerol has not previously been reported for any species of *Trichoderma* and suggests that in *T. hamatum chit42* and *prb1* may be co-regulated by a metabolic pathway which responds to elevated glycerol. As CP-1 is not conserved in *T. atroviride* it may be involved in glycerol response in *T. hamatum.*

Mycoparasitism and glycerol utilisation are examples of alternate carbon source metabolic pathways. In the highly competitive soil environment, glucose is deficient, therefore rapid response to other sources of carbon is a competitive advantage. Competition has been generally considered to play some role in successful biological control by *Trichoderma.* In the field, up-regulation of *chit42* and *prb1* in response to an alternate carbon source, such as glycerol, may have improved the mycoparasitic ability of *T. hamatum* 6Sr4, and provided a competitive edge, which together contributed towards superior biocontrol potential.

Chapter Four
Genetic Variation Within *chit42*, *prb1* and *xbg1.3-110*

4.1 Introduction

Due to the complex nature of eukaryotic gene regulation, identifying the gene(s) responsible for phenotypic variation in fungi is extremely difficult. *Trichoderma hamatum* 6Sr4, S1BYG and 3Sr4-2 showed phenotypic variation in standard biocontrol assays against *Sclerotinia sclerotiorum* and *S. minor* (Chapter 1), however the genetic basis of this variation was unknown.

Various methods have been employed to investigate eukaryotic genomes for genetic markers of phenotypic traits. Genetic fingerprinting techniques, such as UP-PCR and RAPDs are routinely used for detecting genetic variation between fungal isolates. Both techniques amplify non-specific regions of the genome using random primers to generate complex banding patterns which may differ between isolates due to sequence polymorphism. In *Trichoderma*, these methods have been used to aid in taxonomic identification and to identify isolate specific markers for use in field trials (Bulat *et al.*, 1998; Lübeck *et al.*, 1999). In maize, a RAPD marker associated with resistance to *Aspergillus flavus* and aflatoxin production has been described (Guo *et al.*, 2001) and in garlic, RAPD markers associated with resistance to onion white rot have been identified (Nabulsi *et al.*, 2001).

Another approach to studying genetic variation is comparison of the same gene in different isolates. Sequence alignment of the same gene in multiple individuals has revealed genetic variation directly associated with phenotypic traits. In *Drosophila melanogaster*, a polymorphic deletion within the coding region of an antibacterial peptide gene rendered it non-functional (Lazzaro & Clark, 2001). Single nucleotide polymorphisms (SNPs) within promoter regions have been attributed to disease in humans through effects on gene expression (Knight *et al.*, 1999, Berry *et al.*, 1992).

Identifying the source of the genetic variation associated with biocontrol potential may identify qualitative markers for desired traits, or information for genetic modification. *Chit42* and *prb1* are considered key enzymes in mycoparasitism (Cortes *et al.*, 1998) and in *T. hamatum* were upregulated during confrontation with *S. sclerotiorum* (Chapter 3). Accordingly, variation in the structure and function of *chit42* and *prb1* may relate to the variable biocontrol potentials of

6Sr4, S1BYG and 3Sr4-2. The aim of this work was to investigate variation between isolates in *chit42*, *prb1* and *xbg1.3-110* in the nucleotide sequence and level of gene induction. In addition, variation between isolates was investigated using standard UP-PCR fingerprinting protocols (Bulat *et al.*, 1998).

4.2 Materials and Methods

4.2.1 Fungal Isolates

The *T. hamatum* isolates used in this study (6Sr4, S1BYG and 3Sr4-2) were isolated from New Zealand soils (Rabeendran, 2000) and identified as *Trichoderma hamatum* by ITS sequencing (Chapter 2). Isolates were maintained and stored on PDA (Appendix 7.2.1).

4.2.2 Extraction of Genomic DNA for use in PCR Amplifications

Genomic DNA (gDNA) was isolated from frozen mycelium using the Genomag™ kit (Advanced Biotechnologies Ltd.) (Section 2.2.2). Purified DNA was resuspended in 100 µl of sterile water and stored at 4° C. DNA samples were quantified via agarose gel electrophoresis as described in Appendix 7.2.3.

4.2.3 UP-PCR Analysis

UP-PCR analysis was used to assess the level of genetic variation between *T. hamatum* 6Sr4, S1BYG and 3Sr4-2. All PCR amplifications were performed in a Perkin Elmer Model 2400 Thermal Cycler (Perkin Elmer Cetus Corp.). Each 25 µL PCR reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP (Roche Molecular Biochemicals Ltd.), 20 pmoles of each primer, 10 ng of *T. hamatum* DNA and 1.25 U *Taq* DNA polymerase (Roche Molecular Biochemicals Ltd.). The sequence of each UP-PCR primer used and their respective annealing temperature is shown in Table 4.1.

Amplification consisted of an initial denaturation of 5 min at 94°C, followed by 5 cycles of 50 s at 94°C, 2 min at the respective annealing temperature (Table 4.1), and 1 min at 72°C, followed by 34 cycles of 50 s at 94°C, 90 s at the respective annealing temperature, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. PCR products were separated by 1% agarose gel electrophoresis as described in Appendix 7.2.3, except that 200 mL of molten agarose was prepared and poured into a larger (20 cm x 21 cm) gel tray, and products separated at 6V/cm for approximately 3 h. All reactions were fully replicated.

Table 4.1. UP-PCR primers and their respective annealing temperatures.

Primer	Sequence 5'-3'	Annealing Temperature (°C)
Fok1	GGATGACCCACCTCCTAC	52
AS4	TGTGGGCGCTCGACAC	50
L21	GGATCCGAGGGTGGCGGTTCT	55
0.3-1	CGAGAACGACGGTTCT	50
AA2M2	GAGCGACCCAGAGCGG	50
3-2	TAAGGGCGGTGCCAGT	50
L15	GAGGGTGGCGGTTCT	52
AS15	GGCTAAGCGGTCGTTAC	52
AS15inv	CATTGCTGGCGAATCGG	52
L15/AS19	GAGGGTGGCGGCTAG	52
L45	GTAAAACGACGGCCAGT	51

4.2.4 Identification of Polymorphic Sites Within *chit42*, *prb1* and *xbg1.3-110*

A 2.69 kb region of the *chit42* gene, encompassing 1418 bp upstream of the translational start site to 1269 bp of the 1467 bp coding region, was amplified, cloned and sequenced from S1BYG and 3Sr4-2, as described for 6Sr4 (Section 3.2.3).

A 1.187 kb fragment of the 1358 bp coding region of *prb1* was isolated, cloned and sequenced from S1BYG and 3Sr4-2, as described for 6Sr4 (Section 3.2.4.1). (Section 3.2.4.2), The sequence specific primer PRB5-1 (5'-GATAAGGCGCCGGCTACG-3') was designed to the *prb1* sequenced derived in Section 3.2.4.1) and used with the inverse primer I-PRB509 to amplify the *prb1* regulatory region from S1BYG and 3Sr4-2. Amplifications were performed at an annealing temperature of 60°C and resulting PCR products cloned and sequenced as described in Section 3.2.3.

Using primers XBG5'S-2 and XBG5'AS, a 1.8 kb region of the *xbg1.3-110* gene, which encompassed 1 kb upstream from the transcriptional start site, was amplified, cloned and sequenced from S1BYG and 3Sr4-2 as described for 6Sr4 in Section 3.2.5.2.

Combined sequence data from two clones per sequence were aligned revealing multiple single nucleotide polymorphisms (SNPs), however only SNPs present in both clones were considered in further sequence analysis.

4.2.5 Northern Analysis of *chit42* Expression in 6Sr4, S1BYG and 3Sr4-2

To investigate variation in *chit42* gene expression, 6Sr4, S1BYG and 3Sr4-2 were grown in pilot-scale submerged culture and direct confrontation assays as described in Sections 3.2.6 and 3.2.7. In the submerged culture assay, isolates were grown in MM + 2% glycerol only, and at each sampling interval, observations made on the conidial germination. Growth rates on MM agar + 0.2% glycerol were determined over 4 d for each isolate, and distance between *T. hamatum* and *S. sclerotiorum* inoculation plugs adjusted accordingly in the direct confrontation assay. Northern hybridisation analysis was done on total RNA from the assays as described in Section 3.2.8. Assays were performed once only.

4.3 Results

4.3.1 Genetic Variation Within *Trichoderma hamatum*

UP-PCR analysis demonstrated genetic variation between 6Sr4, S1BYG and 3Sr4-2. In eleven separate UP-PCR amplifications (Table 4.1), 6Sr4 and 3Sr4-2 shared banding patterns in eight, and S1BYG and 3Sr4-2 in two. Three representative profiles are presented in Figure 4.1. On the basis of banding patterns, 6Sr4 and 3Sr4-2 appeared to be the most genetically similar.

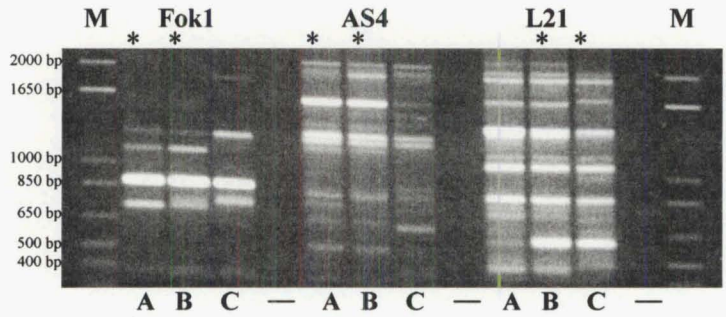


Figure 4.1. UP-PCR amplification of **A:** 6Sr4 **B:** 3Sr4-2 and **C:** S1BYG. Primer sequences are presented in Table 4.1. M = molecular weight marker. Negative controls were included within each set of primers (-). * denotes similar banding pattern

4.3.2 Nucleotide Polymorphism Within *chit42*, *prb1* and *xbg1.3-110*

From the UP-PCR analysis, it was apparent there was variation between the three *T. hamatum* genomes. Sequence analysis revealed many single nucleotide polymorphisms (SNPs) in the coding regions of *chit42* and *prb1*. Of the nine SNPs detected in exonic regions of *chit42*, only four altered the triplet codon such that a different amino acid was encoded. Three of the thirteen exonic SNPs in *prb1* resulted in amino acid substitutions. Amino acid substitutions are

presented in Table 4.2 and hydrophobicity profiles are detailed in Appendix 7.8. The coding region of *xbg1.3-110* was not analysed.

Table 4.2. Amino acid substitutions in *T. hamatum chit42* and *prb1*.

GENE	POSITION	6Sr4	S1BYG	3Sr4-2	DESCRIPTION
chit42	119	Gly	Ala	Ala	Hydrophilic to hydrophobic
	120	Asn	Asn	Thr	No change in charge
	169	Val	Iso	Val	No change in charge
	175	Ala	Ser	Ala	Hydrophobic to hydrophilic
prb1	24	Ala	Thr	Ala	Hydrophobic to hydrophilic
	98	Ser	Asn	Ser	No change in charge
	247	Ser	Gly	Ser	No change in charge

Greater nucleotide variation was observed within the regulatory regions than the coding regions of *chit42* and *prb1*. Nineteen polymorphic sites were identified from the 1310 bp region of the *chit42* promoter and eight in the *prb1* promoter (686 bp). The frequency of SNPs in both promoters was approximately 12 in every 1000 bases. The promoter region of *xbg1.3-110* (939 bp) was also examined for the presence of polymorphisms, revealing nineteen SNPs. The frequency of SNPs occurring was 20 in every 1000 bases, almost double that of *chit42* and *prb1*. Polymorphic sites which interrupted, or lay adjacent to, putative regulatory motifs established in Chapter 3 are presented in Table 4.3 and exact locations of all sites are included in Appendix 7.9.

Table 4.3. Polymorphic sites within the regulatory regions of *T. hamatum chit42*, *prb1* and *xbg1.3-110*.

Gene	Position	6Sr4	S1BYG	3Sr4-2	Feature
chit42	-664	A	G	A	Interupts CP-1
prb1	-315	C	T	C	5' to the *cre1* motif
	-396	T	C	T	Interupts the –397 *AceII* site
	-588	A	T	A	3' to the –575 CCAAT-box motif
xbg1.3-110	-583	G	-	G	3' to the -588 CCAAT-box motif
	-785	A	T	A	3' to the -791 *cre1* motif

4.3.3 Variation in Expression Levels of *chit42*

It was evident from the sequence analysis that some level of polymorphism existed in *chit42*, *prb1* and *xbg1.3-110*, however, on the basis of sequence analysis alone, relevance to gene function and expression could not be determined. To investigate comparative *chit42* gene expression under inducing conditions, two preliminary northern studies were done using the protocols and procedures established in Chapter 3. The results are shown in Figure 4.2. Two major trends were observed in both response to elevated glycerol and confrontation with *S. sclerotiorum*, 6Sr4 was upregulated to a higher level than both S1BYG and 3Sr4-2, and S1BYG *chit42* expression was delayed relative to 6Sr4 and 3Sr4-2.

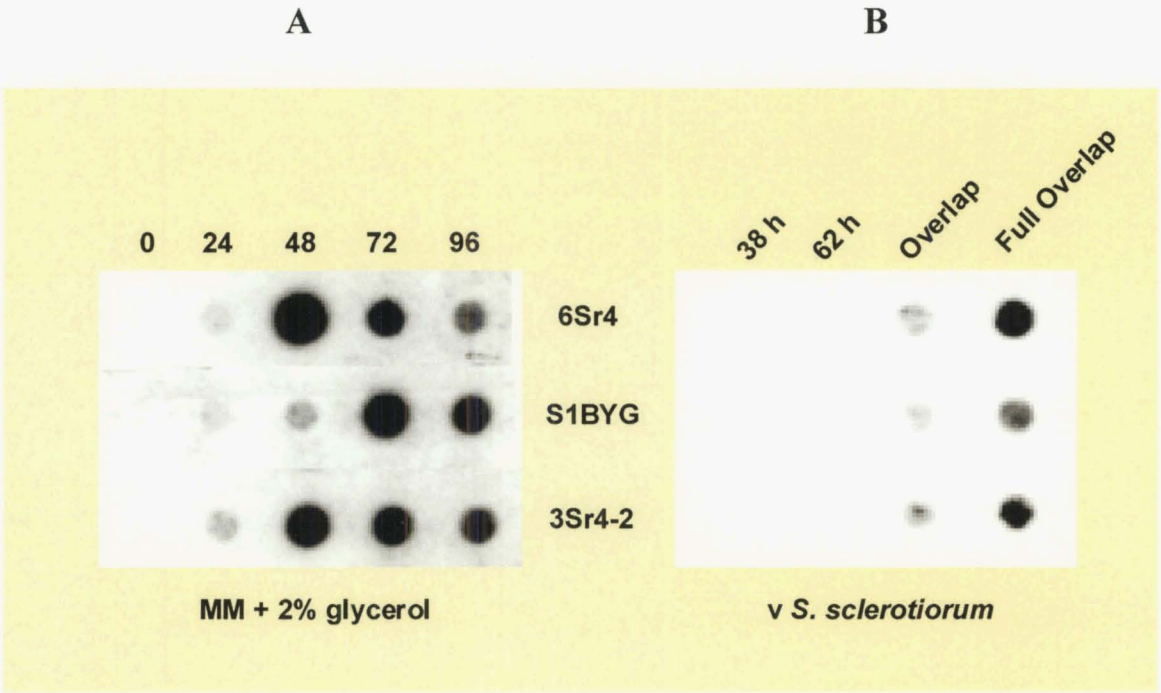


Figure 4.2. *Chit42* expression levels in 6Sr4, S1BYG and 3Sr4-2 under inducing conditions. **A:** Submerged culture assay. **B:** Confrontation assay.

Peak S1BYG *chit42* expression in the submerged culture, at 72 h, coincided with an observed delay in germination. The three isolates germinated at different rates within the submerged culture assay, S1BYG was the slowest. It was not until 72 h that all conidia had germinated in the S1BYG samples.

4.4 Discussion

Both UP-PCR patterns and sequence analysis revealed greater similarity between 6Sr4 and 3Sr4-2 than either shared with S1BYG, which was supported by northern analysis of chit42 expression. This was surprising as 6Sr4 had previously demonstrated superior biocontrol potential (Rabeendran, 2000), and was therefore predicted to show greater variation compared to the isolates of lesser potential. In addition, colony morphology (Chapter 2) suggested S1BYG and 3Sr4-2 to be more similar than 6Sr4.

4.4.1 Nucleotide Variation Between 6Sr4, S1BYG and 3Sr4-2

Whilst some amino acid substitutions in Chit42 and Prb1 resulted in variation in charge, this did not affect the overall hydrophobicity profile. In addition, none of the substitutions interrupted active or glycosylation sites. Nevertheless, the possibility exists that variation within the coding region may have affected protein function, in particular the two substitutions involving glycine. Glycine residues allow unusual polypeptide chain conformations, therefore presence (or absence) may affect protein structure and hence function (Matthews & van Holde, 1990).

Greater variation was observed in the regulatory regions of *chit42*, *prb1* and *xbg1.3-110* than in the amino acid sequences of Chit42 and Prb1. This was not unexpected as the nucleotide identity in the promoter sequences was significantly lower than the amino acid identity between *T. hamatum* and *T. atroviride.* The higher level of polymorphism in the regulatory regions was likely due to lower functional constraints, and indeed more SNPS were identified in the less active upstream regions of the promoter than within the first 200 bp in both *chit42* and *xbg1.3-110.*

Greater polymorphism was observed in the regulatory region of *xbg1.3-110* than *chit42* and *prb1*, which suggested *chit42* and *prb1* to be under higher functional constraint. Chit42 and Prb1 are key enzymes in mycoparasitism, an important mechanism of carbon assimilation in the soil environment, therefore major differences in gene expression as a result of promoter disruptions may be selected against. Conversely, *xbg1.3-110* may be simpler involving fewer crucial nucleotides.

In human studies, some single nucleotide polymorphisms have been demonstrated to have a direct effect in gene expression by altering the protein binding patterns within the regulatory region at the site of polymorphism, or in some cases affecting binding to motifs up to 100 bp

distal (Berry *et al.*, 1992). Many SNPs were identified in *chit42*, *prb1* and *xbg1.3-110*, however sequence analysis alone was insufficient to determine effect, if any, on relative expression levels.

4.4.2 Expression of *chit42* in three *T. hamatum* Isolates

Slower response to elevated glycerol by S1BYG may have been due to the CP-1 disruption within the *chit42* regulatory region. CP-1 was present in 6Sr4 and 3Sr4-2, and postulated to be involved in response to elevated glycerol by binding to an unknown regulatory protein (Chapter 3). Polymorphism within this identity may affect protein binding efficiency, thereby altering gene expression levels under inducing conditions.

Alternately, the delay in S1BYG *chit42* expression may have been due to a comparatively slower germination rate, 24 and 48 h mycelial samples contained a proportion of young mycelium (>24 h). In 6Sr4, a 48 h time lag from germination to maximum *chit42* expression has been observed, therefore mycelium less than 24 h old in the S1BYG samples would have effectively diluted the proportion of *chit42* mRNA within the total RNA. At each time interval, cultures were washed and rinsed through miracloth to remove ungerminated conidia, however it is possible total RNA was also diluted with conidial RNA.

The lower expression levels of *chit42* in S1BYG during direct confrontation may be related to biocontrol potential, however transformation studies have shown *chit42* deletion mutants to have little effect on biocontrol activity (Carsolio *et al.*, 1999). The combined action of multiple enzymes is required for biocontrol activity, therefore a reduction in *chit42* expression alone would not necessarily affect overall biocontrol ability. Further work needs to be done to determine if other mycoparasitism-implicated genes are also induced at a lower rate during confrontation.

Whilst polymorphisms within *chit42* and *prb1* may affect expression levels, the variation in expression observed within *chit42* was likely insufficient to affect overall biocontrol potential. Sequence analysis of *T. hamatum* (Chapter 3) supports the hypothesis of a global inducer of mycoparasitism (Cortes *et al.*, 1998). If the source of variation in biocontrol potential between 6Sr4, S1BYG and 3Sr4-2 lies in the superior mycoparasitic ability of 6Sr4, then the genetic basis of this variation may lie within the global inducer.

Chapter Five
Trichoderma hamatum 6Sr4 as a Model for Studies in the Regulation of Mycoparasitism

One of the major limitations to widespread implementation of biological control has been inconsistency in field performance. One approach towards consistency is to evaluate each BCA in an ecological context for parameters conducive to successful biocontrol. This has lead to usage recommendations for commercial *Trichoderma* BCAs (www.tricho.com). Fundamental research into the genetic basis of biocontrol is another approach. Elucidating the regulatory 'switches' involved in biocontrol mechanisms, such as mycoparasitism, may identify molecular targets for manipulation. Genes implicated in mycoparasitism by *Trichoderma* species are regulated by many different pathways in response to environmental signals, many of which can be simulated *in vitro*. For instance, Mach *et al.* (1999) demonstrated *T. atroviride chit42* expression after prolonged carbon starvation, and in response to physiological stress, and in *T. hamatum chit42* and *prb1* are strongly induced in response to high glycerol (Chapter 3). Some genes have been characterised, however a complete understanding of the molecular mechanisms involved in mycoparasitism is still lacking. This study provides further insight into the regulation of genes involved in mycoparasitism and identifies molecular targets for manipulation of a commercially available *Trichoderma hamatum* biocontrol agent.

Mycoparasitism by *Trichoderma* species has been postulated to involve a global inducer molecule. Similarities in the regulatory regions of *T. hamatum chit42* and *prb1* (this study) support the existence of a global regulator of mycoparasitism (Cortes *et al.*, 1998). Mutational analysis of *T. hamatum* 6Sr4 *chit42* and *prb1* promoter regions may provide further support, however stronger evidence will come from the isolation and characterisation of proteins bound to the promoter during mycoparasitism. A novel yeast-based selection method (Saloheimo *et al*, 2000) has been developed for cloning of positive regulatory genes in *T. reesei*, and this could be applied to *T. hamatum* 6Sr4. In addition, the putative global regulator binding motif MYC3 lay within a highly conserved (90%) 22 bp region of *chit42* and *prb1* promoters. If MYC3 is present in the promoters of other genes involved in mycoparasitism, it may be possible to design primers based on this conservation to find those genes. Identification of novel genes would greatly enhance our understanding of the genetic basis of mycoparasitism, thus identifying molecular targets for manipulation of biocontrol in the field.

Genes implicated in mycoparasitism by *T. atroviride* are known to be under the control of carbon catabolite repression and more recently, nitrogen catabolite repression (Carlos Cortes, pers. comm.). This study supports the prediction of a *prb1* GATA motif at approximately –100, which is actively involved in nitrogen repression. To further support the GATA hypothesis, nitrogen repression assays should be done on *T. hamatum* to determine if *prb1* is regulated as in *T. atroviride*. Understanding the importance of nitrogen repression to mycoparasitism may lead to application recommendations for *Trichoderma* BCAs. If biocontrol activity is reduced in the presence of inorganic nitrogen, due to repression of key genes implicated in mycoparasitism, then growers should avoid applying inorganic nitrogen prior to or just after application of a *T. hamatum* or *T. atroviride* BCA.

Regulatory motifs identified through sequence analysis may be due to statistical probability rather than active function, however alignment with orthologues from other species may predict active binding sites, such as the *prb1* GATA. The upstream regions of *T. harzianum chit42* and *prb1* (Section 3.2.8.2) should be sequenced and aligned to *T. hamatum* and *T. atroviride*, and nitrogen repression studies done on *prb1* if the conserved GATA site is present. If nitrogen catabolite repression of genes implicated in mycoparasitism is conserved within the *Trichoderma* genera, as carbon catabolite repression is, then the recommendation regarding nitrogen application, above, may be extended to all commercial *Trichoderma* BCAs. All regulatory motifs were aligned based on a TSP determined by identity with other *Trichoderma* species. Using RT-PCR a TSP should be conclusively identified for all genes identified in this study.

In *T. hamatum*, *chit42* and *prb1* were strongly induced in elevated glycerol by an unknown regulatory pathway not present or active in *T. atroviride* or *T. harzianum* (Chapter 3). Incorporation of glycerol into *T. hamatum* inoculant formulations may optimise *chit42* and *prb1* gene expression during mycoparasitism in the soil. Field studies could then be conducted to determine amendment effects on biological control, which may lead to recommendations for inoculant formulations designed to optimise biocontrol potential. Combining this approach with recommendations for BCA application relative to the pathogen and host interaction is likely to provide consistent performance from commercial biocontrol agents.

Induction of *chit42* and *prb1* has been demonstrated in response to a range of carbon sources other than glucose, such as xylose, lactose and mannose (*chit42*), and chitin (*prb1*).

The strong induction of *T. hamatum chit42* and *prb1* in response to alternate carbon sources is therefore, likely not limited to glycerol. In addition, due to its liquid state, glycerol would only be suitable for amendment of wet formulations, such as slurries. Therefore, gene expression studies should be done with *T. hamatum* 6Sr4 in response to a range of other alternate carbon sources, to identify other inducers of *chit42* and *prb1*, more suitable for amendment of dry formulations.

Beta-1,3- and 1,6-glucanases have been implicated in mycoparasitism by *Trichoderma* on the basis of induction during growth on media supplemented with autoclaved pathogen cell walls, however gene expression during confrontation has not reported. This study found no *xbg1.3-110* expression during confrontation (Chapter 3) and questions its importance in mycoparasitism. The absence of *xbg1.3-110* expression in 6Sr4 may have been due to the choice of pathogen, *S. sclerotiorum*, therefore gene expression should be investigated in response to confrontation with a range of pathogens, including basidiomycetes and oomycetes.

Cellulases, in particular CBH2, have been strongly implicated in mycoparasitism of oomycetes by *Trichoderma*. Mycoparasitism of *Aphanomyces euteiches* by *T. hamatum* 6Sr4 has been observed, therefore CBH2 may play a role. CBH2 should be cloned and sequenced from 6Sr4, and expression during confrontation with *A. euteiches* examined.

The genetic basis of variation in biocontrol potential by three *T. hamatum* isolates was not able to be determined from this study, however there may be differences in the rate of *chit42* expression. A fully replicated submerged culture assay should be done to determine if the observed differences in the pilot study can be reproduced, though the assay design should be modified to alleviate germination rate effects. A major criticism of northern analysis is that gene expression does not necessarily correlate to protein production and/or secretion, however the time constraints of this study did not allow for protein studies to be done. Differences in the *chit42* expression rate did not necessarily correlate to increased enzymatic activity of Chit42 and Prb1 in the medium, therefore extracellular enzymatic activity of both enzymes should also be investigated.

Relative competitive ability may have contributed towards variation in biocontrol potential between the three *T. hamatum* isolates. In comparison to S1BYG and 3Sr4-2, *T. hamatum* 6Sr4 germinated faster in, and had a faster growth rate on, minimal media (Chapter 4),

which suggested a competitive advantage in a low nutrient environment. Competition usually plays a role in successful biocontrol by *Trichoderma* species (Heljord and Tronsmo, 1998), therefore it is likely that competitive ability, and possibly a faster rate of expression of genes implicated in mycoparasitism, contributed to the superior biocontrol potential of 6Sr4.

In summary, *T. hamatum* 6Sr4 was established as a model for studies in the genetic regulation of mycoparasitism. Characterisation of three genes implicated in mycoparasitism from 6Sr4 provided supporting evidence for two current hypotheses in genetic regulation, and identified molecular targets for optimisation of biocontrol potential. Future work on these targets is likely to lead to usage recommendations for commercial *Trichoderma* BCAs. In addition, preliminary investigation into the genetic basis of variation in biocontrol potential, suggested differences in expression rates of key enzymes implicated in mycoparasitism, and suggested high competitive ability as a defining morphological trait of high biocontrol potential. This work contributes to current knowledge on gene regulation and has potential practical applications. Much work needs to be done before a full understanding of the regulation of mycoparasitism is achieved, and true optimisation of commercial BCA products can be achieved.

Chapter Six
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Chapter Seven
Appendices

7.1 Companies Cited in This Thesis

Advanced Biotechnologies Ltd., Surrey, UK

Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK

Beckton Dickson & Co., Sparks MD, USA

Bio-Rad Laboratories, Hercules CA, USA

Calbiochem, La Jolla, CA, USA

E-C Apparatus Corp., New York, USA

Eppendorf-Netherler-Hinz GmbH, Hamburg, Germany

Germantown (New Zealand) Company, Auckland, New Zealand

Invitrogen Corporation, Carlsbad, CA, USA

Kodak (Australia) Pty., Ltd., Coburg, Vic., Australia

Lynnon Biosoft, Quebec, Canada

Merck New Zealand Limited, Palmerston North, New Zealand

Molecular Bioproducts, San Diego, CA, USA

Nalge Nunc International., Naperville, IL, USA

Perkin Elmer Cetus Corp., Norwalk, Connecticut, USA

Polaroid (UK) Ltd., St Albans, Hertfordshire, England

Promega Corporation, Madison WI, USA

Roche Molecular Biochemicals, Mannheim, Germany

Sigma-Aldrich Pty., Ltd., Castle Hill, NSW, Australia

Thermo Hybaid, Ashford, Middlesex, UK

7.2 Methods

Unless otherwise stated, all chemicals are from Merck New Zealand Ltd. (BDH New Zealand).

7.2.1 Fungal Isolate Maintenance and Storage

Potato-dextrose Agar (PDA) (1 L)

39 g	PDA (Beckton Dickson & Co.)
1 L	H_2O

Autoclaved at 121° C for 15 min at 15 p.s.i.

Fungal isolates were cultured on PDA in Petri dishes at 22°C under 12 h light/dark cycles. For each isolate, 5 mm agar plugs from the actively growing colony margin were transferred to 10 mL PDA slopes in Universal bottles, incubated for 2 d under the same conditions as above, then sealed with parafilm and transferred to 4°C for storage.

7.2.2 Genomic DNA Extraction

Potato-dextrose Broth (PDB) (1 L)

24 g	PDB (Beckton Dickson & Co.)
1 L	H_2O

Autoclaved at 121° C for 15 min at 15 p.s.i.

Harvesting of Mycelium

Mycelial plugs (5 mm diameter) from the stored slope cultures were transferred to PDA in Petri dishes and incubated for 3 d at 22°C under 12 h light/dark cycles. A 5 mm agar plug from each actively growing colony margin was transferred to a deep Petri dish containing approximately 20 mL of PDB and incubated as above for 3 d. Mycelium was harvested by pressing between Miracloth (Calbiochem) and paper towels to remove excess liquid, wrapping in aluminium foil and snap freezing in liquid nitrogen. All mycelial samples were stored at -80°C until required.

Phenol/Chloroform Extraction of DNA from Solution

To a DNA sample in a solvent resistant centrifuge tube, 1 volume Tris-buffered phenol (Invitrogen Corp.) and 1 volume chloroform was added and the solution mixed by gentle inversion. Tubes were centrifuged at 12 000 x*g*, the upper aqueous phase removed to a fresh centrifuge tube and the remaining liquid discarded. If the interphase was chalky, the procedure was repeated until it became clear. To extract residual phenol, 2 volumes chloroform was added, tubes gently inverted and centrifuged at 12 000 x*g*. The upper aqueous phase containing DNA and salts was removed to a fresh tube and the remaining liquid discarded.

7.2.3 Agarose Gel Electrophoresis

50X TAE Buffer (Sambrook *et al.*, 1989) (1 L)

242 g	Tris base (Invitrogen Corp.)
57.1 mL	Glacial acetic acid
100 mL	0.5 M EDTA (pH 8.0)

Made up to 1 L with H_2O

Autoclaved at 121°C for 15 min at 15 p.s.i.

6 X Loading Buffer (Sambrook *et al.*, 1989)

0.25 % Bromophenol blue

0.25 % Xylene cyanol FF

40 % (w/v) Sucrose in water

500 μL aliquots stored at 4°C

1% Agarose Gel (Sambrook *et al.*, 1989)

A 1% agarose gel was prepared by dissolving 1 g of agarose (Agarose MP, Roche Molecular Biochemicals) in 100 mL of 1 X TAE buffer by boiling. The dissolved agarose was cooled to 60°C, poured into a gel casting tray (10 cm x 6.5 cm). containing a well-forming comb and allowed to set for ~1 h. The comb was removed and the casting tray containing the solidified gel transferred to an electrophoresis tank (E-C Apparatus Corp.) containing 1 X TAE buffer.

DNA Quantification

To quantify genomic DNA, 2 μL aliquots mixed with 3 μL 6 X loading buffer and 13 μL water were loaded into individual wells of a 1% agarose gel. A 2 μL aliquot of DNA High Mass™ Ladder (Invitrogen Corp.) prepared in the same manner was loaded into an adjacent well. DNA was separated by electrophoresis at a constant electric voltage (12.5 V/cm) for ~50 min. The gel was stained by submerging in 0.5 μg mL^{-1} ethidium bromide for ~30 min. DNA was visualised on a transilluminator and a polaroid photograph taken using Polaroid 667 instant film (Polaroid [UK] Ltd.). DNA was quantified by visual comparison of sample DNA intensity with that of the DNA High Mass™ ladder.

PCR Product Size Determination

To estimate molecular weight of PCR products, 5 μL aliquots were mixed with 1 μL 6 X loading buffer and separated by agarose electrophoresis as described above. A 5 μL aliquot of a 1 kb Plus DNA Ladder™ (Invitrogen Corp.) was separated in an adjacent well. PCR product size was estimated by position in the gel relative to the known molecular weight bands in the ladder.

7.2.4 Cloning of PCR Products

SOC Medium (Promega Corp.) (100 mL)

2 g	Bacto-tryptone (Beckton Dickson & Co.)
0.5 g	Yeast extract (Beckton Dickson & Co.)
100 mM	NaCl
400 mM	KCl
40 mM	$MgCl_2$ (filter-sterilised)
20 mM	Glucose (filter-sterilised)

The bacto-tryptone, yeast extract, NaCl and KCl were dissolved in 95 mL H_2O and autoclaved at 121° C for 15 min at 15 p.s.i.. The $MgCl_2$ and glucose were added and the total volume brought up to 100 mL with sterile H_2O. The medium was then filter sterilised to 0.2 μm and stored in 1 mL aliquots at -20°C until required

Blue/White Selection Agar (Promega Corp.)

LB agar

10 g	Bacto-tryptone (Beckton Dickson & Co.)
5 g	Yeast extract (Beckton Dickson & Co.)
5 g	NaCl
15 g	Agar (Germantown [NZ] Co.)
1 L	H_2O

Autoclaved at 121°C for 15 min at p.s.i.

1000X Ampicillin Stock

500 mg	Ampicillin (Roche Molecular Biochemicals)
5 mL	H_2O

Filter sterilised to 0.45 μm

Aliquots (500 μL) aliquots at -20°C

X-gal Stock

Dissolved 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Roche Molecular Biochemicals) in 2 mL N,N'-dimethyl-formamide (Sigma-aldrich Pty., Ltd.) in a sterile Universal and covered with aluminium foil to omit light. X-gal stock was stored at -20°C.

Ampicillin was added to the LB agar, just prior to pouring, to give a final concentration of 100 μg mL^{-1}. Using a glass spreader, 20 μL of the 50 mg mL^{-1} X-gal stock was spread over the surface of dry LB/ampicillin plates and allowed to absorb for 10 min prior to use.

7.2.5 Bacterial Maintenance and Storage

LB Broth

As per LB agar recipe above (7.2.4) except agar was omitted

Using a sterile wire loop, single bacterial colonies were inoculated into 25 mL flasks containing 5 mL of LB broth plus ampicillin at 100 μg mL^{-1}. Flasks were shaken overnight at 200 rpm in a 37°C shaking incubator. One mL of culture fluid was transferred to a screw-top centrifuge tube and cells pelleted at 7000 x*g*. The supernatant was removed and cells were resuspended in 1 mL sterile 40% glycerol. Tubes were stored at -80°C.

7.2.6 Northern Hybridisation

20 x SSC

3 M	NaCl
0.3 M	*tri*-Sodium Citrate

Salts were dissolved in H_2O, DEPC-treated and autoclaved at 121° C for 15 min at p.s.i.. Solution was stored at room temperature.

Hybridisation Buffer

0.5 M	NaCl
5%	Blocking Reagent (ECL kit, Amersham Pharmacia Biotech UK Ltd.)

Salt and blocking reagent was mixed immediately in ECL Gold buffer and stirred at room temperature for 1 h. Solution was incubated at 42°C for 1 h with intermittent agitation to dissolve the blocking reagent. Aliquots (25 mL) were stored at -20°C

Primary Wash Buffer

6 M	Urea
0.4%	SDS (Roche Molecular Biochemicals)
0.5X	SSC

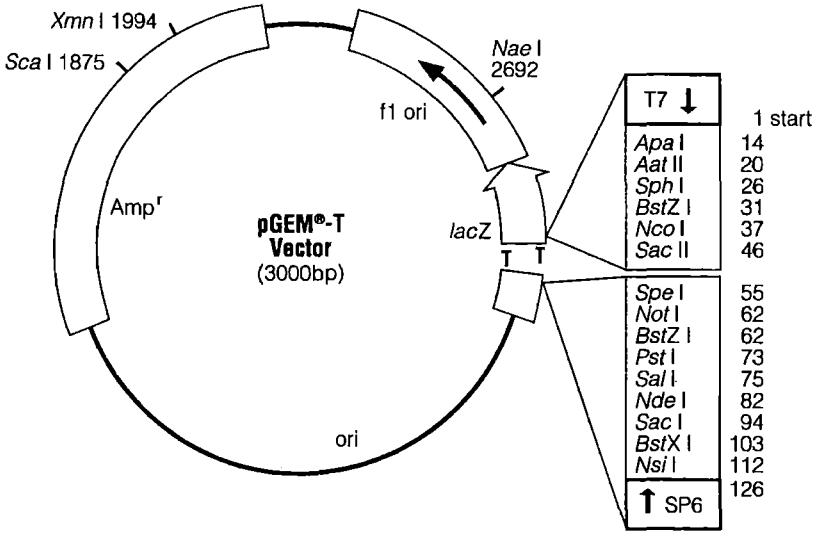
Ingredients were dissolved in 2 L H_2O for about 2 h at room temperature and solution stored for up to 3 months at 4°C.

7.3 Codon Preference

Codon preference of *Trichoderma hamatum chit42* gene exon5 (ascn#: U88560).

T	C	A	G	
	Phe 36	Ser 32	Tyr 38	Cys 100	T
T	Phe 64	Ser 24	Tyr 62	Cys 0	C
	Leu 0	Ser 5	** 0	** 0	A
	Leu 19	Ser 0	** 0	Trp 100	G
	Leu 33	Pro 29	His 20	Arg 0	T
C	Leu 19	Pro 50	His 80	Arg 43	C
	Leu 4	Pro 21	Gln 27	Arg 43	A
	Leu 26	Pro 0	Gln 73	Arg 0	G
	Ile 24	Thr 20	Asn 4	Ser 3	T
A	Ile 71	Thr 60	Asn 96	Ser 35	C
	Ile 6	Thr 20	Lys 0	Arg 14	A
	Met 100	Thr 0	Lys 100	Arg 0	G
	Val 50	Ala 34	Asp 43	Gly 38	T
G	Val 50	Ala 44	Asp 57	Gly 24	C
	Val 0	Ala 13	Glu 0	Gly 38	A
	Val 0	Ala 9	Glu 100	Gly 0	G

7.4 pGEM®-T



7.5 Clonal Map of *xbg1.3-110*

Blue = degenerate PCR

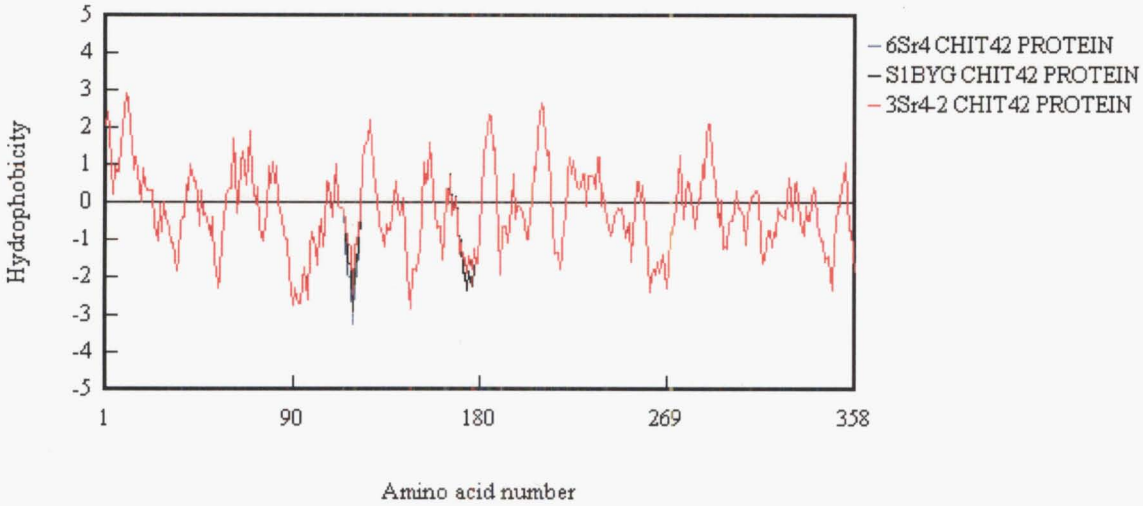
Red = inverse PCR

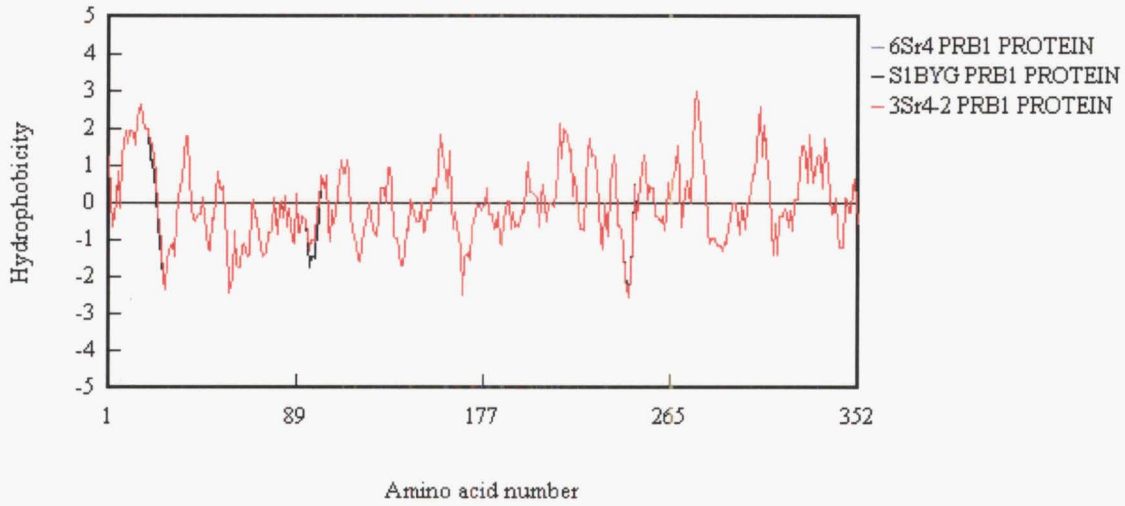
Green = specific PCR

7.7 Base Composition of Identities Between the Regulatory Regions of *T. hamatum* 6Sr4 *chit42*, *prb1* and *xbg1.3-110*

Identity	Composition 5'-3'	Position *chit42*	Position *prb1*	Position *xbg*
CP-1	ATTAGAGCT	-669	-679	
CP-2	GCATGG	-536	-582	
CP-3	TGGCAT	-526	-579	
CP-4	AACGTT	-354	-407	
CP-5	TGGGCACGG	-229	-257	
CP-6	TTCTAG	-176	-204	
CP-7	TTGGCA	-134	-161	
CP-8	GCTTCA	-87	-115	
CP-9	TTGACT	-39	-60	
CX-1	GGAGAC	-192		-212
CX-2	TGGGTT	-123		-127
CX-3	TCCTGC	-25		-11
PX-1	GAAATCG		-102	-95
PX-2	ATTTAAG		-67	-29

7.8 Hydrophobicity Profiles of *chit42* and *prb1*

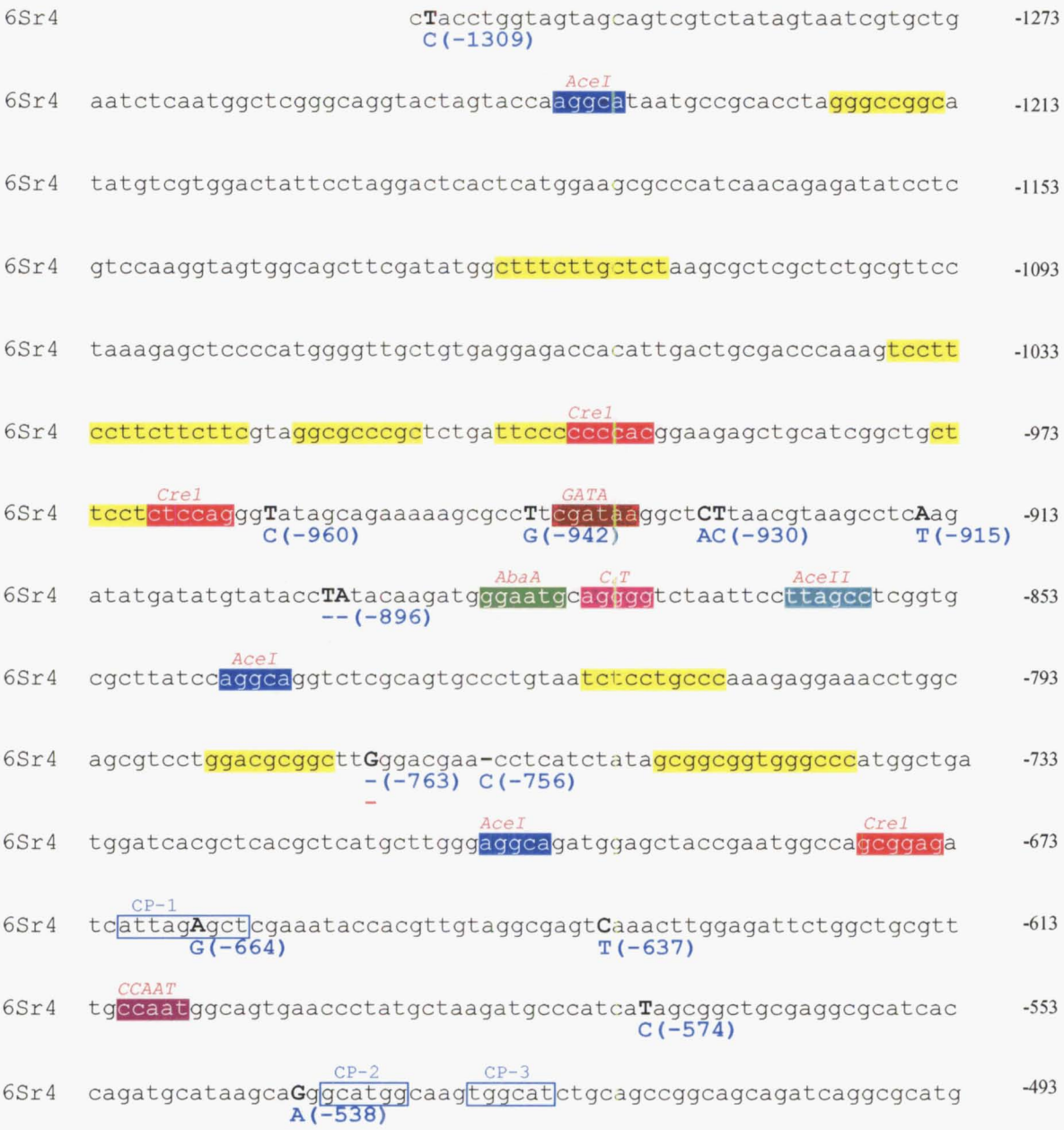


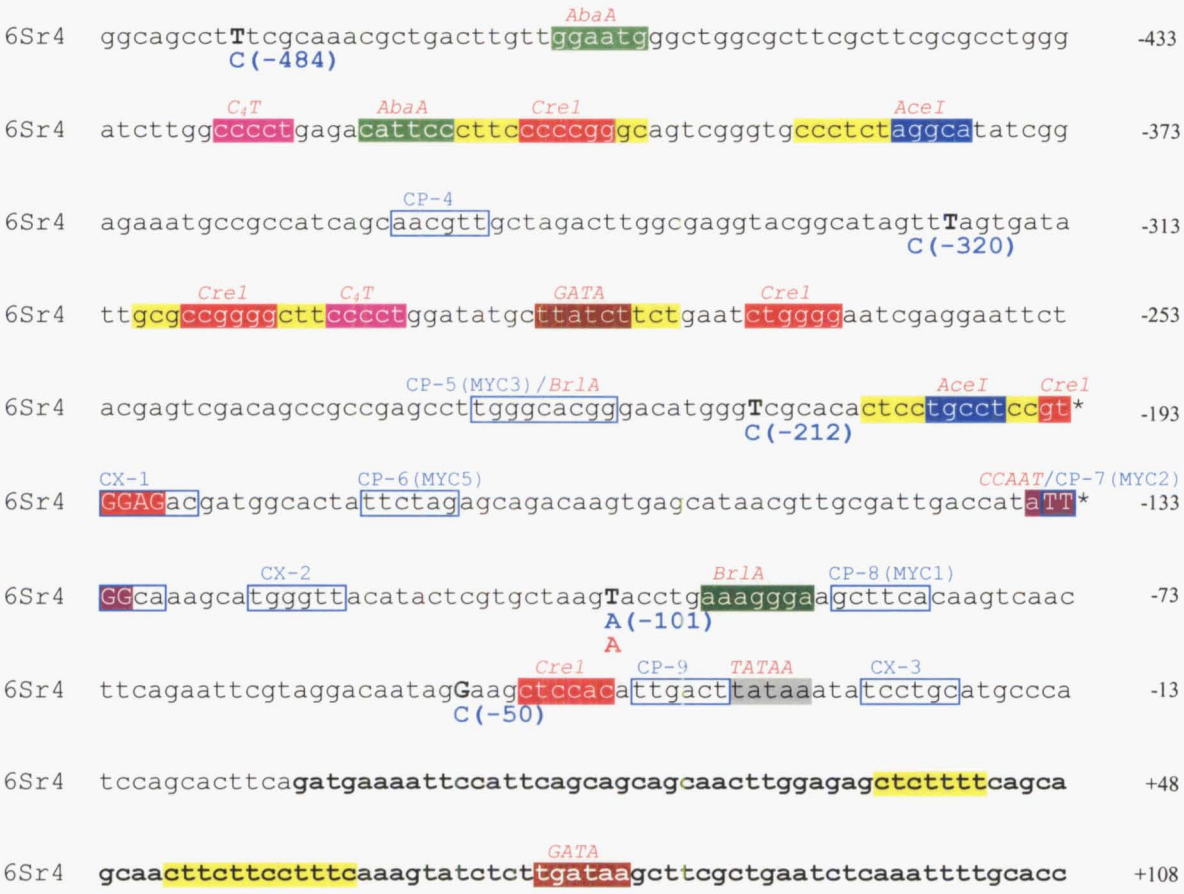


7.9 Location of Single Nucleotide Polymorphisms within the Regulatory Regions of *chit42*, *prb1* and *xbg1.3-110*

Changes in S1BYG are indicated in blue, changes in 3Sr4-2 are indicated in red. Sequence identities between genes are boxed in blue. Numbering is relative to the TSP +1. The 5' untranslated region is in bold. GC- and CT-rich elements are shaded in yellow. Where two motifs overlap, bases are in upper case.
* motif continues on next line

chit42

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6Sr4 ggcagcctTtcgcaaacgctgacttgttggaatggctggcgcttcgcttcgcgcctggg -433
C(-484)
C4T AbaA Cre1 AceI
6Sr4 atcttggccccctgagacattccttccccgggcagtcgggtgccctctaggcatatcgg -373
CP-4
6Sr4 agaaatgccgccatcagcaacgttgctagacttggcgaggtacggcatagttTagtgata -313
C(-320)
Cre1 C4T GATA Cre1
6Sr4 ttgcgccggggcttccccctggatatgcttatcttctgaatctggggaatcgaggaattct -253
CP-5(MYC3)/BrlA AceI Cre1
6Sr4 acgagtcgacagccgccgagccttgggcacgggacatgggTcgcacactcctgcctccgt* -193
C(-212)
CX-1 CP-6(MYC5) CCAAT/CP-7(MYC2)
6Sr4 GGAGacgatggcactattctagagcagacaagtgagcataacgttgcgattgaccataTT* -133
CX-2 BrlA CP-8(MYC1)
6Sr4 GGcaaagcatgggttacatactcgtgctaagTacctgaaagggaagcttcacaagtcaac -73
A(-101)
A
Cre1 CP-9 TATAA CX-3
6Sr4 ttcagaattcgtaggacaatagGaagctccacattgacttataaatatcctgcatgccca -13
C(-50)
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prb1


CP-1
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CCAAT
AceII CP-2/3(MYC1)/CCAAT
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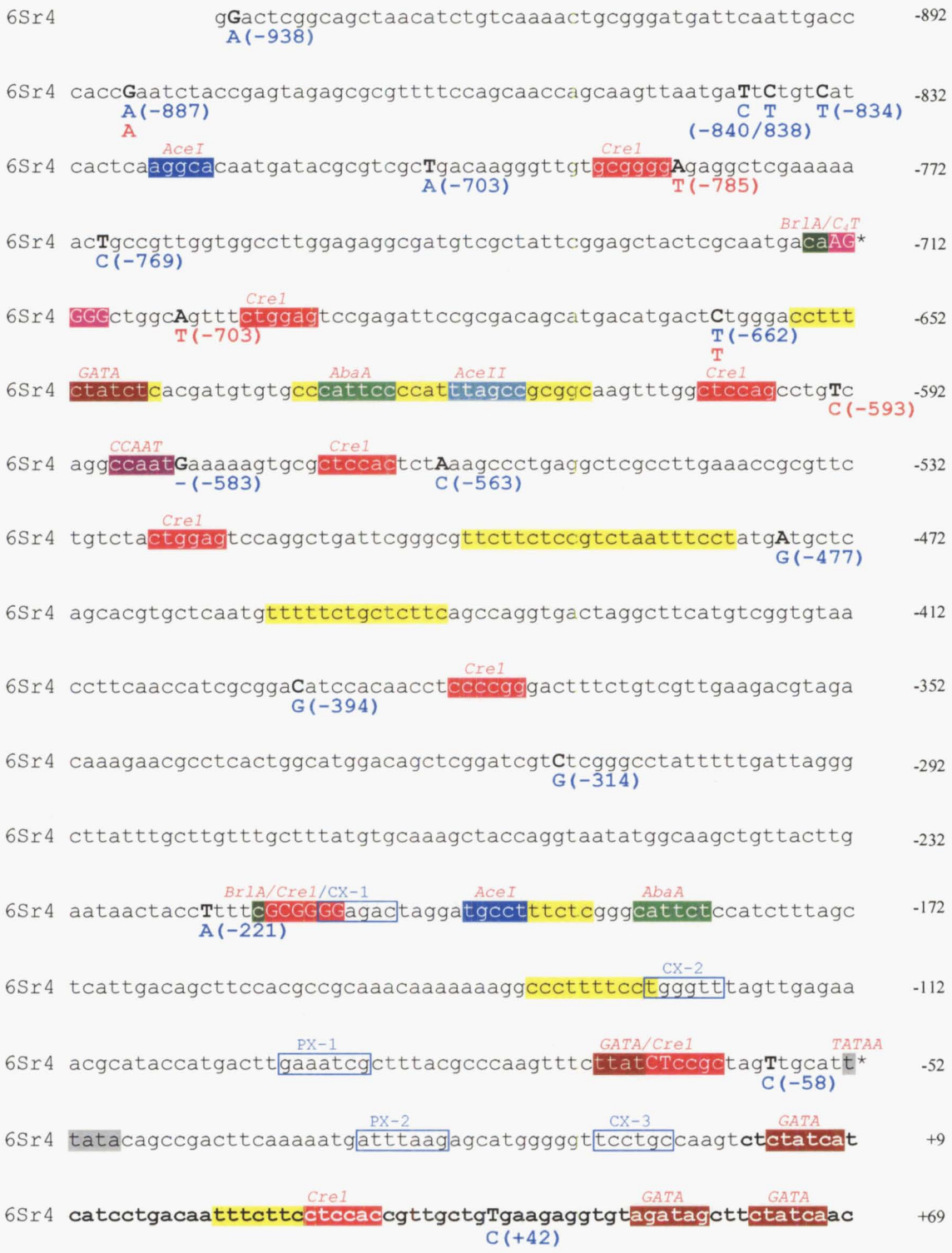
GATA
6Sr4 AtattattatctgtagcacaagcagctcatagagtgttcgcttttaagcgtaaattagcA -511
T(-570)
G(-511)
G
6Sr4 gattttagtttatagatgtcaaataaccttaaaagaatcttaaaattcatttcttcggca -451
CP-4
AceII
6Sr4 tttgttaccgtgtgtccaggagcgatgaagcgatatataatagaacgtttggattagcca -391
C(-396)
6Sr4 tgacgagaatcaaaatatccattttaggaattagatcacgtttaactcatccatatcgac -331
Cre1/GATA
6Sr4 atccgagttggaaaCctggAGatagtgttgctttatggttctgctttccgcattcaaact -271
T(-316)
CP-5(MYC3)
6Sr4 agctgccaagagctgggcacggcggctgccgtaacacaaacatatagtcttggtgtgatc -211
CP-6(MYC5)
AceI
GATA
CP-7(MYC2)
6Sr4 tgttcgttctaggtctttttaggcagcttggttatctataCtcagagacttggcacgcaa -151
A(-170)
CP-8(MYC1)/GATA
PX-1
6Sr4 ttcgcaatagattgatgctggtagacgtgatggaggcttcAgatagcgaaatcgacTttC -91
C
T(-94/-91)
PX-2
CP-9
6Sr4 gatccgcagtgatgatgaaaactatttaagttgactacattcgcagaaaatatttggtaa -31
6Sr4 atcagagcagcaatcaaattctcatttcaActcctccattatataaagcacatcacaaca +30
T(-1)

xbg1.3-110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A(-887)
A
C T T(-834)
(-840/838)
AceI
Cre1
6Sr4 cactcaaggcacaatgatacgcgtcgcTgacaagggttgtgcgggggAgaggctcgaaaaa -772
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T(-785)
BrlA/C4T
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Cre1
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G(-314)
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BrlA/Cre1/CX-1
AceI
AbaA
6Sr4 aataactaccTtttcGCGGGGGagactaggatgccttttctcgggcattctccatctttagc -172
A(-221)
CX-2
6Sr4 tcattgacagcttccacgccgcaaacaaaaaaaggccctttttcctgggtttagttgagaa -112
PX-1
GATA/Cre1
TATAA
6Sr4 acgcataccatgacttgaaatcgctttacgcccaagtttcttatCTccgctagTtgcattt* -52
C(-58)
PX-2
CX-3
GATA
6Sr4 tatacagccgacttcaaaaatgatttaagagcatgggggttcctgccaagtctctatcatt +9
Cre1
GATA
GATA
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